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The Mechanism of Phorbol Ester-Induced Tumor Promotion

by

Zhimin Lu

A dissertation submitted to the Graduate Faculty in Biology in Partial fulfillment of the requirement for the degree of Doctor of Philosophy
The City University of New York

1998

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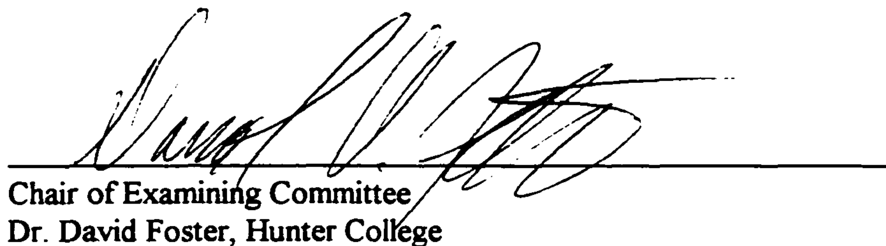

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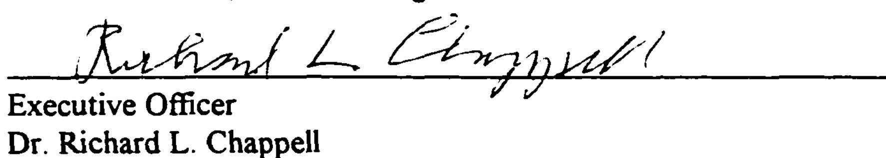

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ABSTRACT

The Mechanism of Phorbol Ester-Induced Tumor Promotion

by

Zhimin Lu

Advisor: Dr. David A. Foster

Tumor promoting phorbol esters activate, but then deplete cells of protein kinase C (PKC) with prolonged treatment. It is not known whether phorbol ester-induced tumor promotion is due to activation or depletion of PKC. In rat fibroblasts overexpressing the c-Src protooncogene, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induced anchorage-independent growth and other transformation-related phenotypes. The appearance of transformed phenotypes induced by TPA in these cells correlated not with activation, but rather with depletion of expressed PKC isoforms. Consistent with this observation, PKC inhibitors also induced transformed phenotypes in the c-Src-overexpressing cells. Bryostatin 1, which inhibited the TPA-induced downregulation of the δ PKC isoform specifically, blocked the tumor-promoting effects of TPA, implicating PKC

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δ as the target of the tumor promoting phorbol esters. Consistent with this hypothesis, expression of a dominant negative PKC δ mutant in cells expressing c-Src caused transformation of these cells; and rottlerin, a protein kinase inhibitor with specificity for PKC δ, like TPA, caused transformation of the c-Src-overexpressing cells. These data implicate that the downregulation of PKC δ results in phorbol ester-induced tumor promotion effect.

To look for the mechanism of phorbol ester-induced downregulation of PKC, we investigated the role of the ubiquitin-proteasome pathway in the downregulation of PKC isoforms in response to the tumor promoting phorbol ester TPA. In 3Y1 rat fibroblasts, proteasome inhibitors prevent the depletion of PKC isoforms α, δ, and ε in response to the TPA. Consistent with the involvement of the ubiquitin-proteasome pathway in the degradation of PKC isoforms, ubiquitinated PKC α, δ, and ε was detected within 30 min of TPA treatment. Diacylglycerol, the physiological activator of PKC, also stimulated ubiquitination and degradation of PKC suggesting that ubiquitination is a physiological response to PKC activation. Compounds that inhibit activation of PKC prevented both TPA- and diacylglycerol-induced PKC depletion and ubiquitination. Moreover, a kinase-dead ATP-binding mutant of PKC α could not be depleted by TPA treatment. These data are consistent with a suicide model whereby activation of PKC triggers its own degradation via the ubiquitin-proteasome pathway. Proteasome inhibitors also blocked the tumor promoting effects of TPA on 3Y1 cells overexpressing c-Src,

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which results from the depletion of PKC δ, this provides further evidence to support that the tumor promoting effect of phorbol esters is due to depletion of PKC δ, which has an apparent tumor suppressor function.

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I dedicate this work to my family, especially my loving parents, my brothers, and my beloved wife, Ji Wang. Without their unconditional love, support, and encouragement, I would never have this achievement.

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List of Abbreviations

TPA	12-O-tetradecanoylphorbol-13-acetate
PKC	Protein Kinase C
cPKC	conventional PKC
nPKC	novel PKC
aPKC	atypical PKC
PLD	phospholipase D
PLC	phospholipase C
DG	diacylglycerol
PS	phosphatidylserine
DIC8	1,2-dioctanoyl-*sn*-glycerol
DMEM	Dulbecco's modified Eagle medium
DTT	dithiothreitol
EDTA	ethylenediaminotetraacetic acid
EGF	epidermal growth factor

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CHAPTER I

Introduction

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1. Tumor promotion and phorbol ester

The phenomenon of ***tumor promotion*** illustrates both the possible involvement of a nongenetic mechanism in cancer induction and the synergistic interaction of cancer inducing agents. The initial discovery of tumor promotion in the early 1940s was based on the finding that the skin of an animal when treated with certain compounds(now recognized as DNA-damaging carcinogens), the odds of a tumor appearing could be greatly augmented by further treatment with a second compound having a different structure and chemistry. The first compound which is a DNA-damaging carcinogen is called a ***tumor initiator***, and the second is called a ***tumor promoter***, which is not by itself mutagenic, can cause cancer selectively in skin previously exposed to a tumor initiator (Berenblum, 1974; Darnell et al., 1990) (Figure 1).

In contrast to initiators, promoters do not need to be metabolized to be active , they have no tendency to react as electrophiles, and they rarely induced tumors by themselves. Therefore promoters must act on cells through a mechanism quite different from that of initiators. To be effective, a promoter must be applied repeatedly over many weeks or months and requires previous initiation to be tumorigenic. In the absence of prior initiation, tumor promoters have no permanent effects on cell metabolism, whereas following initiation they can set in motion events that lead to an irreversible alteration in the cell. The end result could be a DNA alteration, or it could be a changed state of cellular differentiation. By unknown mechanisms, a promoter must produce a second permanent alteration in a cell that has already undergone an initiator-induced mutational

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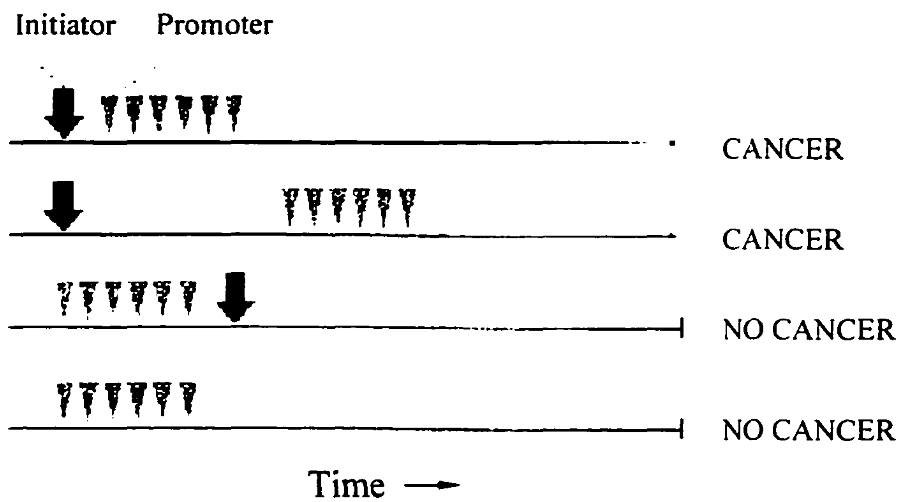

Figure 1. **Some possible schedules of exposure to tumor initiator (mutagenic) and a tumor promoter (nonmutagentic) and their outcomes.**

Cancer ensues only if the repeated exposure to the promoter follows exposure to initiator and only if the intensity of exposure to the promoter exceeds a certain threshold.

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change. Thus tumor promoters act to amplify the effects of initiators (Darnell et al., 1990; Alberts et al., 1994).

The most widely studied tumor promoters are ***phorbol esters***, such as ***12-O-tetradecanoylphorbol-13-acetate (TPA)*** which provides a model for the action of most tumor promoters. A major intracellular receptor for phorbol ester is ***protein kinase C (PKC)***, an enzyme that phosphorylates serine or threonine residues on other proteins. (Castagna et al., 1982; Blumberg, 1988). Phorbol esters can substitute for ***diacylglycerol (DG)*** as a cofactor for PKC and, in association with Ca^{2+} and phospholipids, strongly activate PKC. However, upon prolonged phorbol ester treatment, PKC is proteolytically degraded (Young et al., 1987). The time course for PKC depletion upon phorbol ester treatment varies substantially for different cell types from a few hours to a few days. Tumor promotion requires repeated long term exposure to phorbol esters, suggesting that depletion rather than activation of PKC, is important for tumor promotion. However, it has been pointed out that even though PKC is depleted by prolonged phorbol ester treatment, newly synthesized PKC would be brought to the membrane where there would be a short-lived, but potentially significant, phorbol ester-induced activation of PKC (Liao et al., 1994). Thus it is not clear whether activation or depletion of PKC is important for the tumor promoting effects of phorbol esters.

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2. Protein Kinase C family

Protein Kinase C comprises a multigene family of serine/threonine kinases that play crucial roles in signal transduction and in the regulation of cell growth and differentiation (Nishizuka, 1995). The complexity of the PKC pathway is clearly shown by the fact that PKC represents at least 11 isozymes with different patterns of tissue expression, subcellular localization and cofactor requirements as well as functional diversity (Ohno et al., 1991). On the basis of their biochemical properties and sequence homologies, they have been divided into three groups: the conventional PKCs (cPKC-α, −β1,-β2, and-γ.), the novel PKCs novel (nPKC-δ, −ε, −η, −θ, and -μ), and the atypical PKCs (aPKC-ζ, and -ι, which is the human counterpart of mouse PKC-λ.) (Goodnight et al, 1995). The conventional PKCs are activated in a Ca^{2+}-, DG- and phosphotidylserine-dependent manner. The activation of novel PKCs are Ca^{2+}-independent but DG- and phosphotidylserine-dependent. The atypical PKCs are unresponsive to DG and Ca^{2+}, but responsive to particular inosital phospholipids (Nakanishi et al., 1993).

In all cases, the PKCs consist of an N-terminal regulatory domain and a C-terminal catalytic domain (Figure 2). The catalytic domain has serine/threonine-specific protein kinase activity. In the conventional and novel PKC isozymes, the regulatory domain is thought to inhibit this catalytic activity through a so-called pseudosubstrate region near its N terminus. Adjacent to this pseudosubstrate region is a pair of highly conserved zinc finger structures termed C1 domain, which represent the phorbol ester binding domain and which contribute to the interaction with an essential cofactor,

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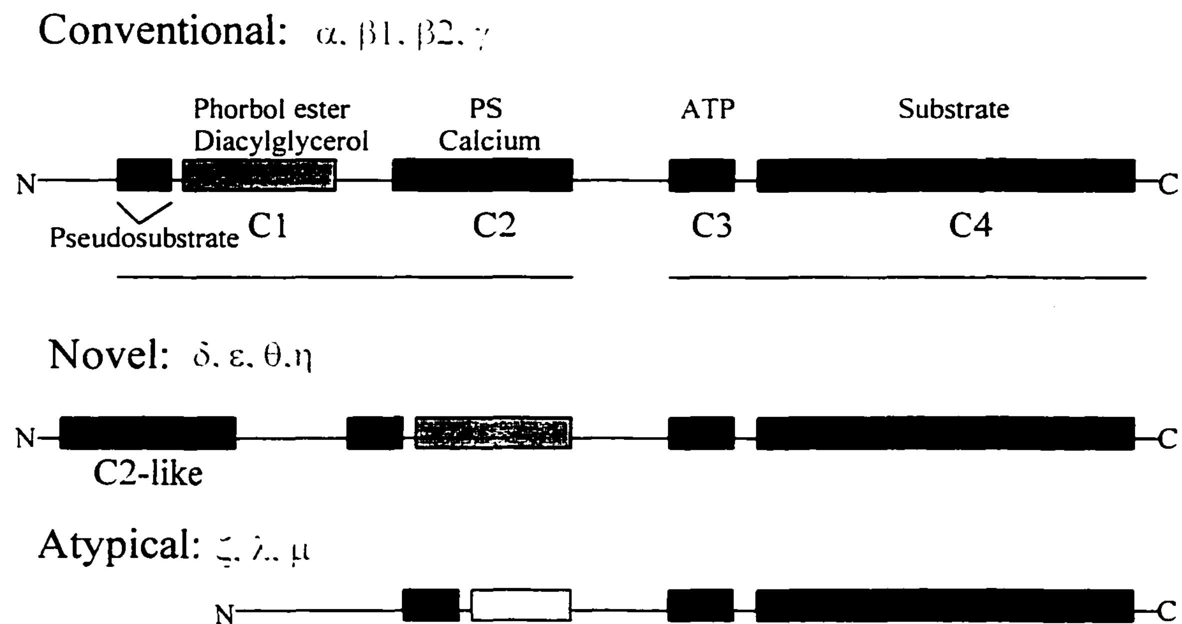

Figure 2. **The schematic structure of PKC isoforms.**

Shown here are the domains of PKC isoforms involved in lipid, phorbol ester, zinc (C1), Ca^{2+} (C2), ATP (C3) and substrate binding (C4). Also indicated a pseudosubstrate region near the N terminus. Ca^{2+}-independent PKC isoforms (nPKC and aPKC) lack the Ca^{2+} binding region. aPKC isoforms only have a truncated lipid binding domain (C1).

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anionic phospholipid (Hurley et al., 1997). In the conventional PKCs, a second domain in the regulatory region, the C2 domain, bestows Ca^{2+} dependence. The novel isozymes lack this region and correspondingly lack Ca^{2+} dependence, although they have a modified C2 homolog N-terminal to the C1 domain (Sossin & Schwartz, 1993). DG, the endogenous activator, and its ultrapotent analogs, the phorbol esters, act as hydrophobic switches upon binding (Zhang et al., 1995), helping to recruit PKC to the membrane (Szallasi et al., 1994), a process referred to as translocation. X-ray crystallography of the PKC δ C1 b domain revealed that the phorbol ester inserts into a hydrophobic surface, promoting interaction of the C1 domain with the membrane. It thus functions as a hydrophobic switch (Zhang et al., 1995).

Not only do the various isozymes show considerable diversity in their structures and regulatory properties, but they have been shown to exhibit different biological effects. PKCα and -ε were shown to inhibit phospholipase C activity (Ozawa et al., 1993), and PKC β and -ε were proven to link the mast cell high affinity receptor IgE to the expression of c-fos and c-jun (Razin et al., 1994). PKCδ and -α participate in TPA-induced myeloid differentiation in 32D cells (Mischak et al., 1993). Overexpression of PKC ε leads to increased cell growth, increased cell density at confluence and induction of anchorage-independent growth in NIH and rat fibroblast. Overexpression of PKC ε in these cells also renders them tumorigenic in nude mice (Mischak et al., 1993; Watanabe et al., 1992). PKCα and -β2 play distinct roles in the transduction of proliferate and differentiating signals in K-562 erythroleukemia cells; PKC α inhibits whereas PKC β2 promotes cell growth (Murray et al., 1993).

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The fact that different PKC isozymes have different or even opposite effects in stimulating and inhibiting cell growth raises another important question. which is that activation or downregulation of which isozymes of PKCs in response to phorbol esters is important for phorbol ester-induced tumor promoting effects.

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CHAPTER II

Materials and Methods

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Cells and Cell Culture Conditions

Rat 3Y1 cells or rat 3Y1 cells expressing either v-Src or c-Src were maintained in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum (HyClone). Cell cultures were made quiescent by growing to confluence and then replacing with fresh media containing 0.5% newborn calf serum for one day. For growth of cells in soft agar, 1 x 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) as described previously (Qureshi et al., 1993). Cells expressing the kinase-dead PKC α were generated as described previously (Lu et al., 1997). The kinase-dead PKC α clone was generated by a mutation to the ATP binding site as described previously (Ueda et al., 1996).

Transfection, G418 or Hygromycin Selection

Cells were plated at a density of 10^5 cells/100 mm dish 18 h prior to transfection. Transfections were performed by using lipofectamine reagent (Gibco) according to the vendors instructions. Transfected cultures were selected in 400 μg/ml G418 or hygromycin 200μg/ml for 10-14 days at 37°C. At that time G418- or hygromycin-resistant colonies picked and expanded for further analysis under selective conditions.

Materials

[^{3}H] thymidine, [γ-^{32}P] ATP, and [^{3}H] myristate were obtained from New England Nuclear. The PKC inhibitors staurosporine, calphostin C, chelerythrine chloride,

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bisindolylmaleimide II, Rottlerin, and Go 6976 were obtained from Calbiochem. Monoclonal antibodies for PKC α, ε and ζ were obtained from Transduction Laboratories; a polyclonal antibody for PKC δ was obtained from Santa Cruz. A monoclonal antibody for ubiquitin was obtained from Zymed. The antiphosphotyrosine monoclonal antibody (PY20) was obtained from Transduction Laboratories. Monoclonal antibody 327 used to immunoprecipitate Src proteins was obtained from Oncogene Science.

Cell lysate preparation and subcellular fractionation.

Cells were grown to approximately 90% confluence in 100 mm culture dishes and were then shifted to DMEM containing 0.5% serum for 24 hours. Cells were washed three times with ice-cold isotonic buffer [phosphate-buffered saline (PBS); 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4, pH 7.2]. For subcellular fractionation, cells from 100 mm dishes were washed and then scraped into 2 ml homogenization buffer (20 mM Tris-HCl [pH 7.5], 5 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM dithiothreitol, 200 μM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cells were then disrupted with 20 strokes in a Dounce homogenizer (type B pestle) and the lysate was centrifuged at 100,000 X g for 1 h. The supernatant was collected as the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton-X100. After incubation for 30 min at 4°C, the suspension was centrifuged at 100,000 g for 1 hour. The supernatant was collected as the membrane fraction. For whole cell lysates, cells were treated with 3 ml

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homogenization buffer containing 1% Triton X-100 followed by centrifugation at 100,000 X g for 1 h. The supernatant was collected and used as the whole cell lysate.

Immunoprecipitation and Western blot analysis.

Extraction of proteins from cultured cells was performed as previously described (Lu et al., 1997) with a modified buffer consisting of 50mM tris-HCl (pH7.5), 1% Triton X-100, 150mM NaCl, 1mM DTT, 0.5 mM EDTA, 0.1 mM phenyl methyl sulfonyl fluoride, leupeptin (12 mg/ml), aprotinin (20 μg/ml), 100 μM sodium vanadate, 100 μM sodium pyrophosphate, 1 mM sodium fluoride, 10 mM ethylmethylmaleimide, and 50 mM hemin. Cell extracts were clarified by centrifugation at 12,000 rpm and the supernatants (1500 μg protein/ml) were subjected to immunoprecipitation with anti-PKC δ, α and ε antibodies. After overnight incubation at 4^0C, protein A-agarose beads were added for an additional 3 hours. Immunocomplexes were then subjected to Western blot analysis as described previously (Lu et al., 1997). Western blot analysis with anti-ubiquitin antibody was performed with modifications described by Avantaggiati et al. (Avantaggiati et al., 1996).

Phospholipase D Assays

Confluent 35 mm culture dishes were prelabeled for 4 h with [^{3}H]-Myristate, 3 μCi (40 Ci/mmol) in 3 ml of media containing 0.5% newborn calf serum. Phospholipase D (PLD) catalyzed transphosphatidylation in the presence of 1% butanol was performed as described previously (Song et al., 1991; Song and Foster, 1993). Extraction and characterization of lipids by thin layer chromatography was performed as previously described (Song et al., 1991).

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DNA Synthesis

Confluent cells were placed in 0.5% serum for 24 h in 24 well tissue culture dishes. DNA synthesis was measured by a one hour pulse with [^{3}H]-thymidine (1 µCi/ml; 20 Ci/mmole). After the one hour pulse, the cells were collected and trichloroacetic acid precipitatable counts were determined by scintillation counting.

Protein Kinase Assays

Protein kinase assays were determined according to procedures described by Clark and Brugge (1993).

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CHAPTER III

Tumor Promotion By Depleting Cells of PKC δ

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INTRODUCTION

Phorbol ester, the best studied tumor promoter, has a dual effect on its major intracellular receptor, protein kinase C. Short term treatment leads to the activation while prolonged treatment results in the downregulation of PKC. PKC is a multigene family consisting of at least 11 isoforms which can be divided into conventional, novel and atypical subcategories. Upon phorbol ester treatment, both conventional and novel PKCs become membrane associated and active while the atypical PKCs are unresponsive to phorbol treatment. Emerging evidence suggests important differences among PKC isozymes both in their regulation and in their biological roles. To investigate the role of activation or depletion of PKC in response to phorbol ester treatment and the role of PKC in phorbol ester-induced tumor promotion process will provide a better understanding of the tumor initiator-promoter model, so that control of cancer could be achieved through control of either initiators or promoters.

We have found that the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates anchorage independent growth of rat fibroblasts overexpressing the c-Src protooncogene. Thus, TPA is able to induce amplification of cells that have an initiating mutation (c-Src overexpression), and is therefore functioning very much like a tumor promoter in this cell culture model. We have exploited the promotion-like properties of TPA on the c-Src-overexpressing rat fibroblasts to investigate the role of PKC in tumor promotion.

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RESULTS

Cells overexpressing c-Src display a transformed phenotype with long term treatment with TPA

Our laboratory has been examining the role of PKC in the transduction of intracellular signals initiated by the tyrosine kinase activity of v-Src (Spangler et al., 1989; Qureshi et al., 1991; 1992; Zang et al., 1995). These studies were extended to cells overexpressing the non-transforming protooncogene c-Src. Upon prolonged exposure to TPA (400 nM, 24 hours), we observed that 3Y1 rat fibroblasts overexpressing c-Src displayed a morphology that resembled that displayed by v-Src-transformed 3Y1 cells (Figure 3A). TPA did not have this effect upon the parental 3Y1 cells (Figure 3A). To determine whether the morphological changes observed in response to TPA actually represented a transformed phenotype, we examined the ability of the TPA-treated c-Src-expressing cells to form colonies in soft agar. As shown in Figure 3B, the c-Src-overexpressing cells that were treated with TPA formed colonies in soft agar, whereas the parental 3Y1 cells did not. The efficiency of colony formation correlated with the levels of c-Src expression in several independent clones of c-Src-overexpressing 3Y1 cells (Figure 3B). We next looked at the effect of TPA treatment on cell cycle regulation in 3Y1 cells and in 3Y1 cells overexpressing c-Src. Cells were grown to confluence and then placed in low serum (0.5%) for 24 h which minimized DNA synthesis as measured by the incorporation of [^{3}H] thymidine. If TPA was added to the confluent c-Src-overexpressing 3Y1 cells, substantial increases in DNA synthesis levels could be detected 12 h after TPA treatment

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and reached peak levels by 20 h (Figure 3C). TPA did not significantly affect DNA synthesis in the parental 3Y1 cells (Figure 3C). We previously demonstrated that both v-Src and v-Ras activate PLD activity (Song et al., 1991; Jiang et al., 1995)). The activation of PLD did not require PKC and in fact, depleting cells of PKC actually appeared to enhance v-Src-induced PLD activity (Song and Foster, 1993). Therefore, we examined the effect of TPA treatment upon the PLD activity in the c-Src-overexpressing cells. In Figure 3D, it is shown that upon prolonged treatment with TPA (24 h, 400 nM), the PLD activity in the c-Src-overexpressing cells was elevated to levels observed previously in v-Src-transformed cells (Song et al., 1991; Song and Foster, 1993). The long term TPA-treatment had no effect upon the PLD activity in the parental 3Y1 cells (Figure 3D). These data are consistent with TPA having promotion-like effects on cells overexpressing c-Src by allowing these partially transformed cells to be amplified under the restricted growth conditions of soft agar suspension, allowing cell cycle progression and inducing a biochemical phenotype characteristic of v-Src-transformed cells.

The transforming effect of TPA on c-Src-overexpressing cells correlates with the depletion of PKC isoforms

To investigate whether the effects of TPA were due to the activation or down-regulation of PKC, the kinetics of appearance of morphological transformation was compared with kinetics of PKC isoform depletion. The kinetics of appearance of the transformed morphology induced by TPA on the c-Src-expressing cells is shown in Figure 4A. The round refractile morphology characteristic of transformed cells could be detected by 6 hours after the addition of TPA. We next examined the kinetics of PKC isoform

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Figure 3. **Cells overexpressing c-Src display a transformed phenotype upon treatment with TPA.**

(A). 3Y1 cells, and 3Y1 cell overexpressing either c-Src or v-Src were either untreated or treated with TPA (400 nM) for 24 hours at which time the morphology of the cells was examined as shown.

(B) Anchorage-independent growth was examined in 3Y1 cells, v-Src-transformed cells and three independent c-Src-expressing 3Y1 cell lines in the presence or absence of TPA (400 nM). 10^3 cells were suspended in soft agar and the percentage of cells forming colonies was determined. The relative levels of c-Src expression, as determined by Western blot analysis, is shown in parenthesis.

(C) 3Y1 cells and 3Y1 cells overexpressing c-Src were grown to confluence and then placed under low serum growth conditions (0.5 %) for 20 hours. TPA (400 nM) was then added and DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine during a one hr pulse 24 hours later. Error bars represent the standard error for triplicate samples from a representative experiment.

(D) PLD activity in 3Y1 cells and 3Y1 cells overexpressing c-Src in the presence and absence of TPA (400 nM) was determined as described previously (Song and Foster, 1993). Error bars represent the range of duplicate samples from a representative experiment after normalization to the PLD activity in the untreated parental 3Y1 cells.

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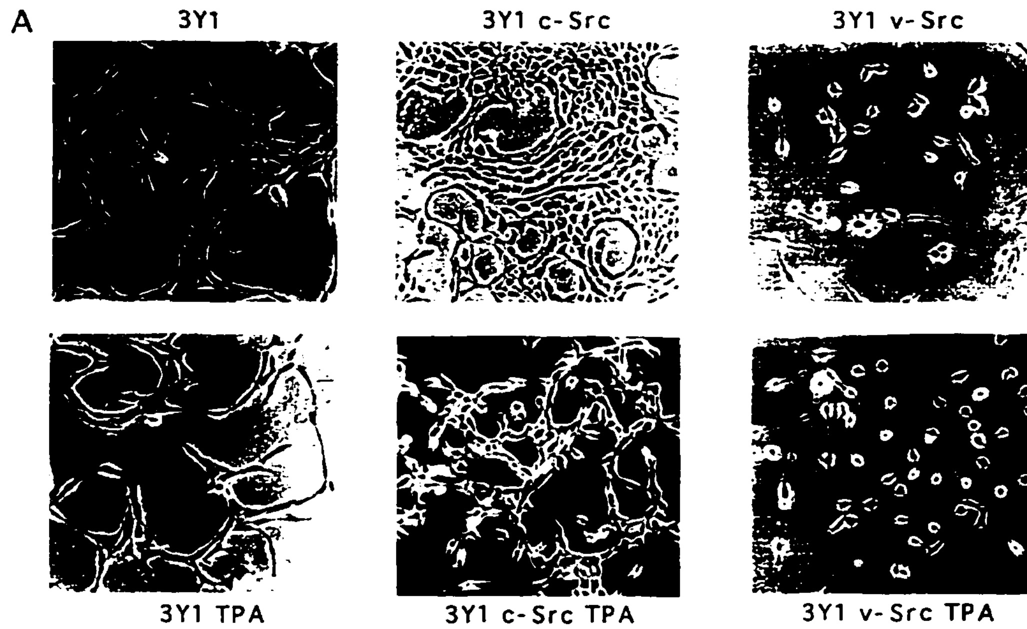

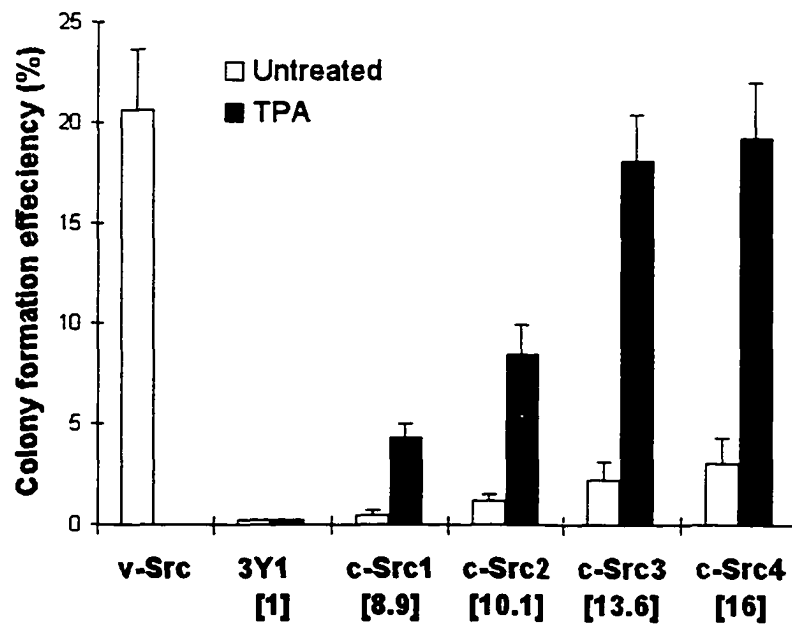

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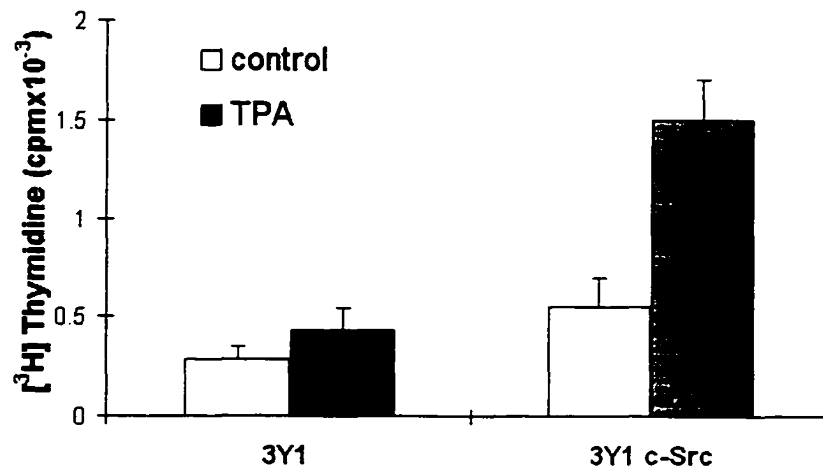

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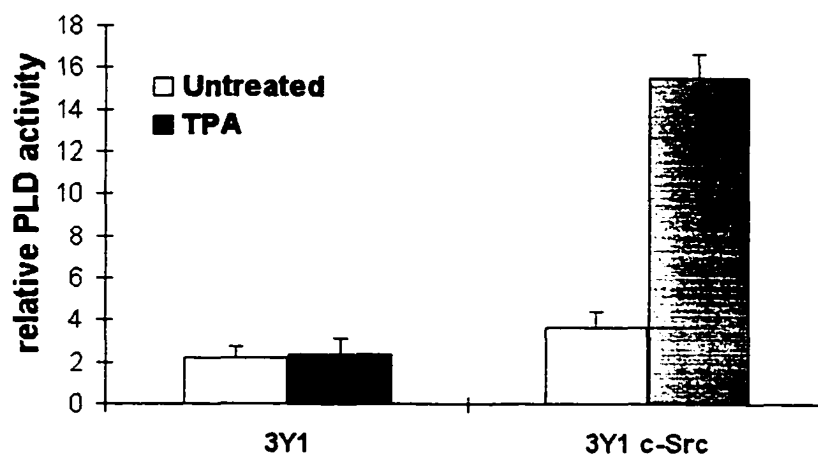

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Figure 4. **The effects of TPA correlate with the disappearance of PKC.**

(A) The kinetics of the transformed morphology observed in Figure 3 was determined by examining the morphology at the times shown.

(B) The level of PKC isoforms α, δ, ε and ζ were determined at the times shown after TPA (400 nM) addition.

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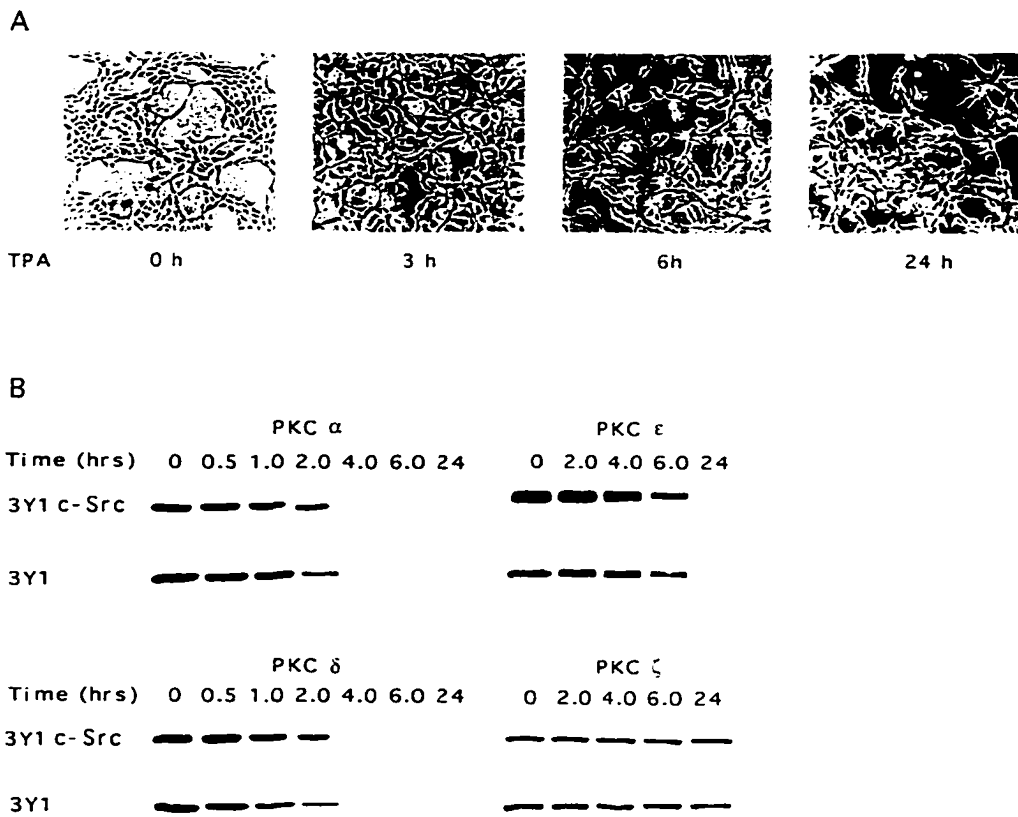

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depletion. We previously reported that in 3Y1 cells, PKC isoforms α, δ, ε and ζ are the predominant isoforms expressed (Zang et al., 1995). As shown in Figure 4B, the α and δ isoforms were dramatically reduced by 4 hours and not detectable by 6 hours after TPA treatment; the ε isoform was substantially reduced by 6 hours, but was noticeably slower in depletion in response to TPA. As expected, the TPA-resistant ζ isoform was unaffected by this treatment. These data reveal a correlation between the appearance of morphological transformation and the down-regulation of PKC in response to TPA with morphological transformation beginning to be detected within 2 hours after depletion of the α and δ isoforms.

TPA treatment does not affect the level or kinase activity of c-Src

We next examined the effect of PKC depletion on c-Src protein levels and kinase activity. We first looked at the effect of TPA treatment on the kinase activity of c-Src, and as shown in Figure 5A, neither c-Src autophosphorylation nor the ability to phosphorylate enolase was affected by TPA treatment of the c-Src-overexpressing cells. Similarly, there were no changes in either the level of c-Src protein (Figure 5B) or the level of cellular tyrosine phosphorylation (Figure 5C) in response to TPA treatment. Thus, the effect of TPA-induced enhancement of transformation does not appear to be due to any obvious direct effects upon either c-Src protein levels or kinase activity.

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Figure 5. **TPA treatment does not affect the level or kinase activity of c-Src.**

The effect of TPA treatment (400 nM, 24 h) on the kinase activity of c-Src (A) c-Src protein levels (B) and total cellular phosphotyrosine (C) was determined as described in the methods section.

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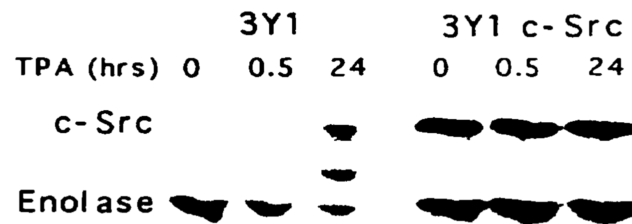
A



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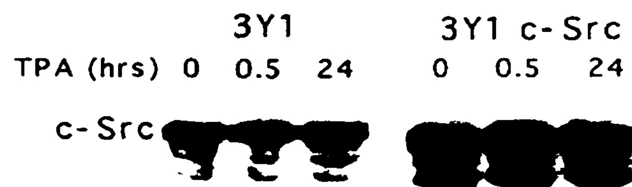

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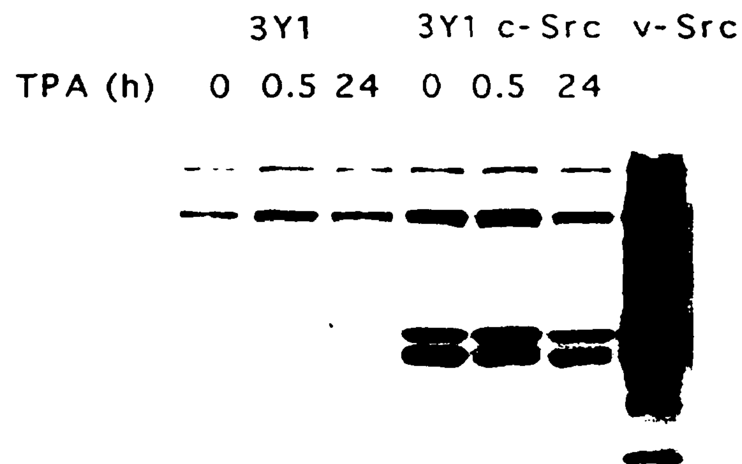

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Inhibiting PKC induces transformed phenotypes in c-Src-expressing cells

The data presented above are consistent with a model in which it is the down regulation of PKC, specifically PKC α and δ Isoforms, being responsible for the promotion-like effects of TPA on the c-Src-overexpressing 3Y1 cells. To further implicate downregulation of PKC as being responsible for the tumor promoting effects of TPA, we examined the effect of compounds that inhibit PKC on 3Y1 cells and 3Y1 cells overexpressing c-Src. We first examined the effect of the PKC inhibitors on DNA synthesis. c-Src-overexpressing and parental 3Y1 cells were treated with several compounds that inhibit PKC by different mechanisms. These included staurosporine, bisindolylmaleimide II, calphostin C, and chelerythrine chloride. Staurosporine and bisindolylmaleimide II are ATP analogs; calphostin C competes with the physiological activator of PKC diacylglycerol; and chelerythrine chloride competes with PKC substrates. As shown in Figure 6A, all four inhibitors mimicked the effect of TPA treatment on DNA synthesis observed in Figure 3. The effect of the inhibitors could be detected within a few hours after treatment with the inhibitors, suggesting that inhibiting PKC was facilitating the release from a late G_1 block. We also investigated the effect of the PKC inhibitors on PLD activity; and, as observed with long term TPA treatment, each of the inhibitors led to increased PLD activity in the c-Src-overexpressing cells (data not shown). We next examined the effect of the PKC inhibitors on the morphology of the 3Y1 cells and the 3Y1 cells overexpressing c-Src. While the effects of most of the PKC inhibitors on cell morphology were subtle and inconclusive, the effects of staurosporine, which was the most potent stimulator of DNA synthesis, were quite striking.

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Staurosporine treatment resulted in a pronounced refractile morphology characteristic of transformation in the c-Src-overexpressing 3Y1 cells, but not on the parental 3Y1 cells. The transformed morphology could be detected between 2 and 4 hours after treatment (Figure 6B). This time course is similar to the appearance of the transformed morphology observed in response to the depletion of PKC, in which PKC α and PKCδ were gone between 4 - 6 hours and the transformed phenotype could begin to be detected between 2 to 4 hours later (see Figure 4). Like the long term TPA treatment, staurosporine had no effect upon the kinase activity of c-Src over the time course used, although longer term treatments were slightly inhibitory (data not shown). Because of the toxicity of these compounds, we were unable to examine the effect of the inhibitors upon anchorage independent growth. While it is difficult to ascertain the precise effects of the PKC inhibitors upon the cells, all of the PKC inhibitors, which inhibit PKC by different mechanisms, induced the transformation-related phenotypes only in the c-Src-overexpressing cells and importantly, did not activate PKC. These data further suggest that depletion, rather than activation, of PKC is responsible for the promoter-like effects of TPA on the c-Src-overexpressing cells.

Bryostatin 1 blocks the tumor promoting ability of TPA

Bryostatin 1 is an activator of PKC that has been reported to prevent tumor promotion in mouse skin by TPA (Hennings et al., 1987). We therefore examined the effects of bryostatin 1 on the TPA-induced transformed morphology in the c-Src-overexpressing cells. We first investigated the effect of bryostatin 1 on the TPA-induced transformed

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Figure 6. **Inhibiting PKC induces transformed phenotypes in c-Src-expressing cells.** (A). 3Y1 cells and 3Y1 cells overexpressing c-Src were grown to confluence and then placed in low serum conditions (0.5%) for 24 h. Staurosporine (7 nM), calphostin C (100 nM), chelerythrine chloride (6 μM) or bisindolylmaleimide II (100 nM) were then added and DNA synthesis was determined 10 h later as described Figure 3E. Since long term treatment with the inhibitors is toxic to the cells, the cells were treated with the inhibitors for 2 h after which the media was changed.

(B) 3Y1 cells and 3Y1 cells overexpressing c-Src were treated with staurosporine (Stauro) (7 nM) as shown and the morphology of the cells was examined 3 hours later.

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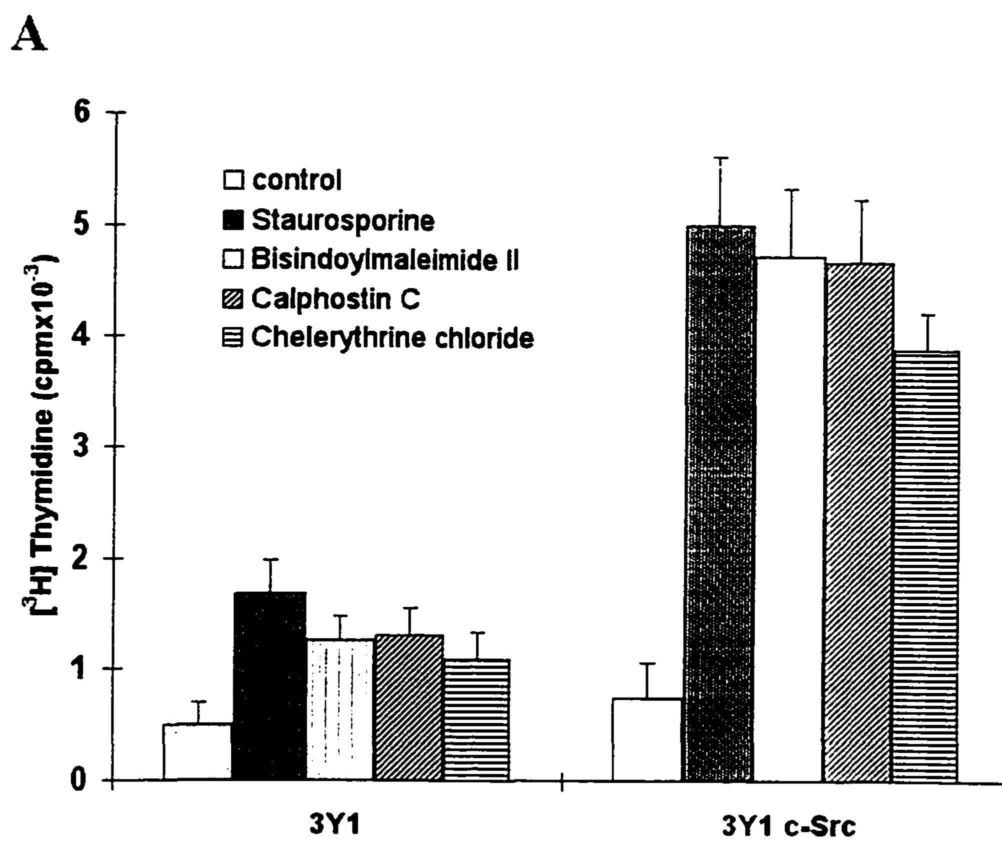
A



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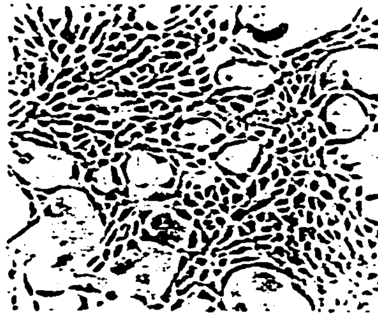
B

Stauro - +

3Y1

3Y1
c-Src



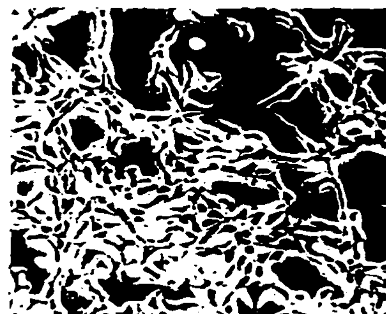


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Figure 7. **Bryostatin 1 blocks the promoter activity of TPA.**

(A) 3Y1 cells overexpressing c-Src were either untreated or treated with TPA (400 nM), bryostatin 1 (Bryo) (1 μM) or both as shown, and the morphology of the cells was examined 24 hr later.

(B) The ability to form colonies form in soft agar was determined as in Figure 1 in the presence of TPA (400 nM) and bryostatin 1 (1 μM) as shown.

(C) The effect of bryostatin 1 on the TPA-induced increase in PLD activity was investigated as described in Figure 3.

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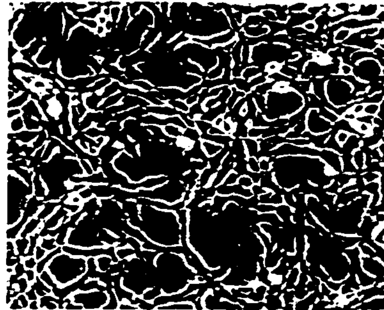
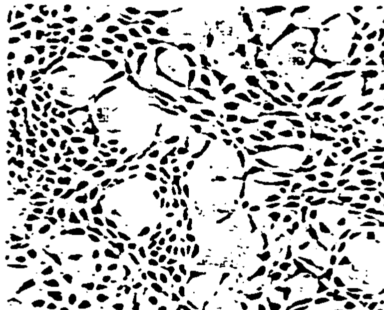
A

Bryo/TPA -/-

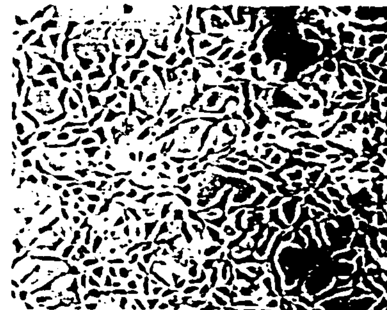


-/+

Bryo/TPA +/-



+/+



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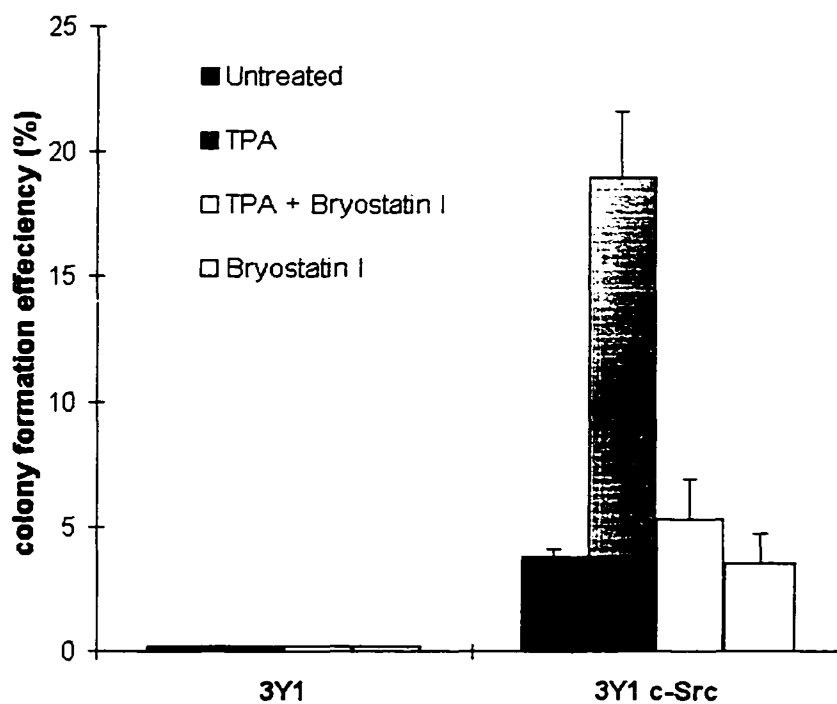

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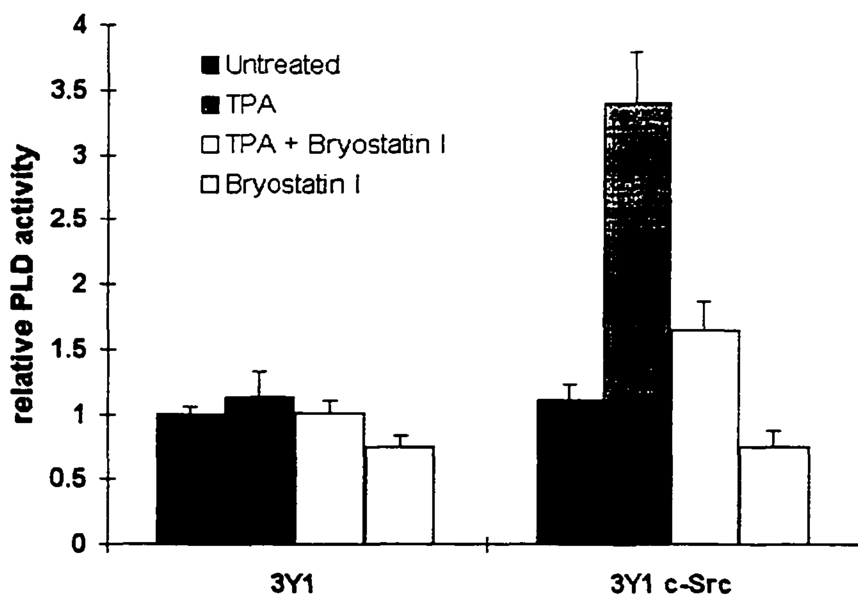

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morphology in the c-Src-overexpressing 3Y1 cells. As shown in Figure 7A, bryostatin 1 completely blocked the TPA-induced refractile morphology. Bryostatin 1 also blocked the TPA-induced colony formation in soft agar observed in the c-Src-overexpressing cells (Figure 7B). These inhibitory effects of bryostatin 1 were not due to toxic effects of this compound since we could culture cells in bryostatin 1 at the concentrations used here for several weeks with no detectable toxicity or changes in growth properties (data not shown). Bryostatin 1 also blocked the TPA-induced increase in PLD activity (Figure 7C) and DNA synthesis (not shown). Thus, as reported previously for TPA-induced tumor promotion in mouse skin, bryostatin 1 inhibits the tumor promotion-like effects of TPA on the c-Src-overexpressing cells.

Bryostatin 1 prevents TPA-induced downregulation of PKC δ, but not PKC α or ε

Differential regulation of PKC isoforms by TPA and bryostatin 1 has been reported (Szallasi et al., 1994a; 1994b). We therefore examined the effect of bryostatin 1 on the level of the PKC isoforms in the c-Src-overexpressing cells that were both treated and untreated with TPA. As shown in Figure 8A, bryostatin 1 prevented the TPA-induced down-regulation of the δ, but not the α or ε PKC isoforms. Also shown in Figure 8A is that bryostatin 1, by itself, lead to down-regulation of the α and ε, but not the δ PKC isoforms; and, as shown above in Figure 7A, this treatment did not induce a transformed morphology. In addition to preventing the TPA-induced morphology changes and PKC δ down-regulation, bryostatin 1 also stimulated membrane association of PKC isoforms α, δ and ε (Figure 8B), which correlates with activation of PKC isoforms. Thus, bryostatin1

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Figure 8. **Bryostatin 1 prevents TPA-induced downregulation of PKC δ, but not PKC α or ε.**

(A) The effect of bryostatin 1 on PKC isoforms α, δ, ε, and ζ in c-Src-overexpressing 3Y1 cells was determined by Western analysis. The cells were untreated or treated with TPA (400 nM) and bryostatin 1 (1 μM) for the times shown.

(B) The effect bryostatin 1 on the cellular distribution of PKC isoforms α, δ, and ε was determined by isolating membrane and cytosolic fractions form the c-Src-overexpressing 3Y1 cells and determing the levels of these PKC isoforms in the two fractions by Western analysis as described previously (Zang et al., 1995).

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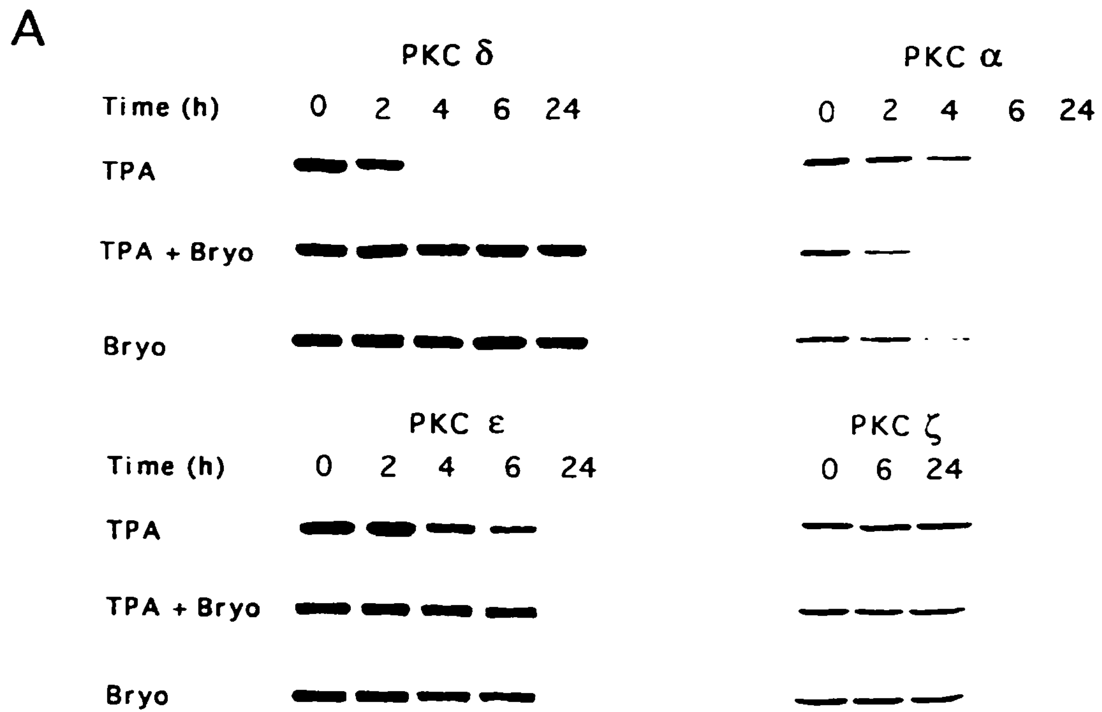

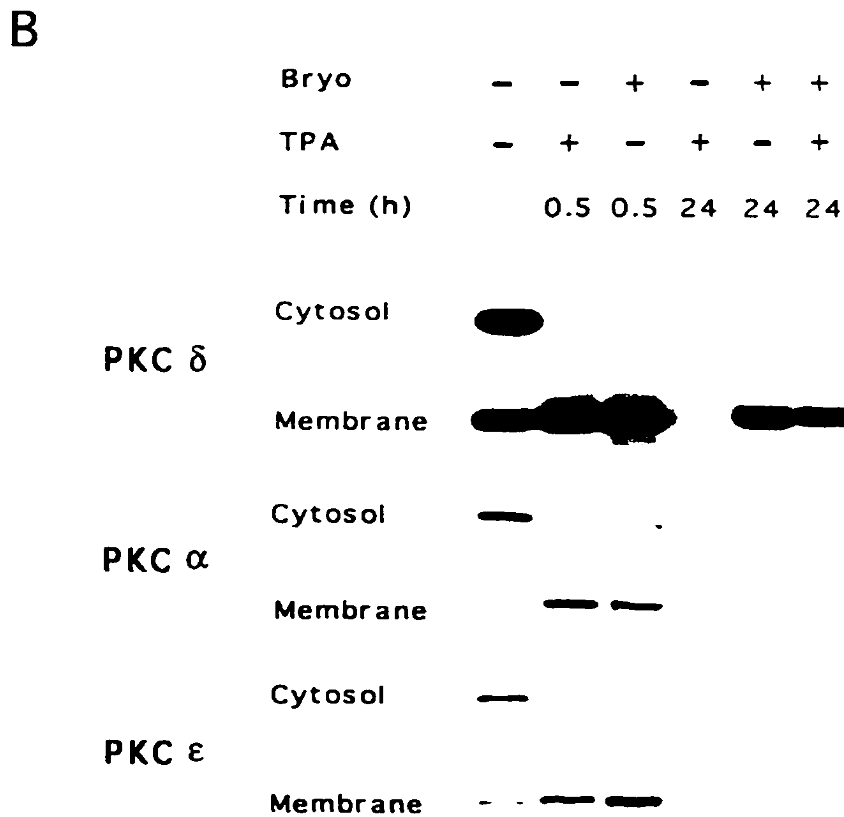

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is capable of both activating and depleting PKC isoforms much like TPA, with the only detectable differences between bryostatin 1 and TPA treatment being that bryostatin 1 is unable to downregulate PKC δ and to induce the transformed phenotypes induced by TPA. And, since bryostatin 1 is able to inhibit the effects of TPA, the data suggest that downregulation of PKC δ accounts for the promoter-like properties of TPA on the c-Src-overexpressing 3Y1 cells.

The effects of bryostatin 1 on TPA-induced transformation in c-Src-overexpressing cells correlates with the level of PKC δ

To further establish the relationship between the presence of PKC δ and the TPA-induced transformed phenotype, we examined the dose response to bryostatin 1 in c-Src-overexpressing cells. In Figure 9A, it is shown that even at 1 nM bryostatin 1, cells begin to look less transformed and that by 100 nM, the cells are essentially flat and resemble the cells that had not been treated with TPA. The effect of increasing concentrations of bryostatin 1 on the levels of PKC δ are shown in Figure 9B where it can be seen that inhibition of the transformed morphology observed in Figure 9A correlates strongly with the appearance of PKC δ.

A dominant negative PKC δ mutant and a PKC δ-specific inhibitor induces transformation of cells overexpressing c-Src

The data presented above implicate PKC δ as the basis for the tumor promoting-like effects reported here. To establish that PKC δ inhibition was sufficient for the tumor

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Figure 9. **The effects of bryostatin 1 on TPA-induced transformation in c-Src-overexpressing cells correlates with the level of PKC δ.**

c-Src-overexpressing 3Y1 cells were treated with TPA (400 nM) and increasing concentrations of bryostatin 1 (Bryo) as shown and the morphology (A) of the cells and PKC δ levels (B) were examined 24 hours later.

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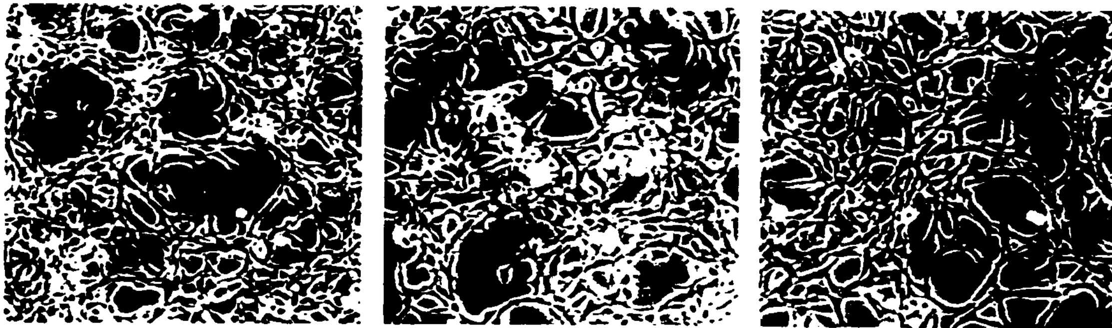
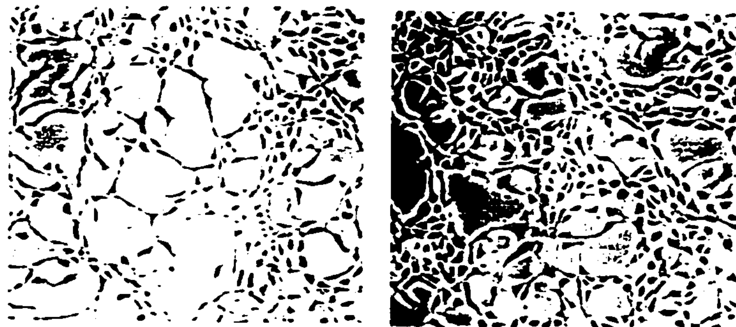
A

TPA/Bryo



+/- +/+ 1nM +/+ 100nM



+/+ 1uM - / -

B

TPA	+	+	+	+	−
Bryo (nm)	0	1	100	1000	0

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promotion-like effects in the c-Src-overexpressing cells, we inhibited the effects of PKC δ specifically. We first introduced a dominant negative PKC δ mutant into the c-Src-overexpressing cells. This mutant (DK376A), which has a Lys to Ala mutation in the conserved ATP-binding site in PKC δ was shown previously to act as a dominant negative mutant for PKC δ (Hirai et al., 1994), as was a similar mutation where Lys 376 was converted to Arg (Li et al., 1995). Upon transfection of a plasmid expressing the dominant negative PKC δ mutant into the c-Src-overexpressing cells, we selected several clones and then examined the ability to form colonies in soft agar. As shown in Figure 10A, these cells now formed colonies in soft agar and there was a correlation between colony number and the level of PKC δ. Expression of the dominant negative PKC δ mutant also caused morphological transformation (Figure 10B).

We next examined the effect of compounds reported to inhibit the α and δ PKC isoforms preferentially. Unlike the less-specific PKC inhibitors used in Figure 6, these inhibitors were not toxic to the cells and could therefore be used to examine anchorage-independent growth. Go 6976 is highly specific for the Ca^{2+} -dependent PKC isoforms which includes PKC α, but not PKC δ or ε (Martiny-Baron et al., 1993); and Rottlerin preferentially inhibits the δ PKC relative to the α and ε isoforms (Gschwendt et al., 1994). The effect of these compounds on colony formation in soft agar is shown in Figure 10C. The PKC α inhibitor had no effect upon colony forming ability; however, the PKC δ inhibitor induced colony formation to levels that were higher than those induced by TPA. The effect of rottlerin occurred in the presence of either bryostatin 1 or Go 6976. Rottlerin also induced a transformed morphology in the c-Src-overexpressing

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cells (Figure 10B). Interestingly, rottlerin induced colony formation of the parental 3Y1 cells; however, the colony-forming efficiency was half that observed for the c-Src-overexpressing cells and the colonies grew only to about 20% of the size of the colonies for c-Src-overexpressing cells (data not shown). Thus, downregulation of PKC δ may be able to promote tumor formation in systems other than the c-Src-overexpressing system described here. These data demonstrate that inhibition of PKC δ is sufficient for induction of anchorage independent growth of cells overexpressing c-Src.

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Figure 10. **A dominant negative PKC δ mutant and a PKC δ-specific inhibitor induces transformation of cells overexpressing c-Src.**

(A) c-Src-overexpressing cells were transfected with a plasmid expressing a dominant negative PKC δ mutant. Several clones were picked and Western analysis was used to examine PKC δ expression levels as shown. The ability to form colonies in soft agar of the parental c-Src-overexpressing cells and 3 dominant negative expressing clones was examined as described in Fig . 3.

(B) Cells overexpressing c-Src were treated with bryostatin 1 (Bryo) (1 μM), rottlerin (30 μM), Go 6976 (0.5 μM) as shown and the morphology of the cells was examined 24 hours later. The morphology of c-Src-overexpressing cells that were stably transfected with a dominant negative PKC δ mutant (clone 3) is also shown.

(C) c-Src-overexpressing 3Y1 cells were examined for the ability to form colonies in soft agar in the presence of TPA (400 nM), bryostatin 1 (Bryo) (1 μM), rottlerin (30 μM), Go 6976 (0.5 μM) as shown.

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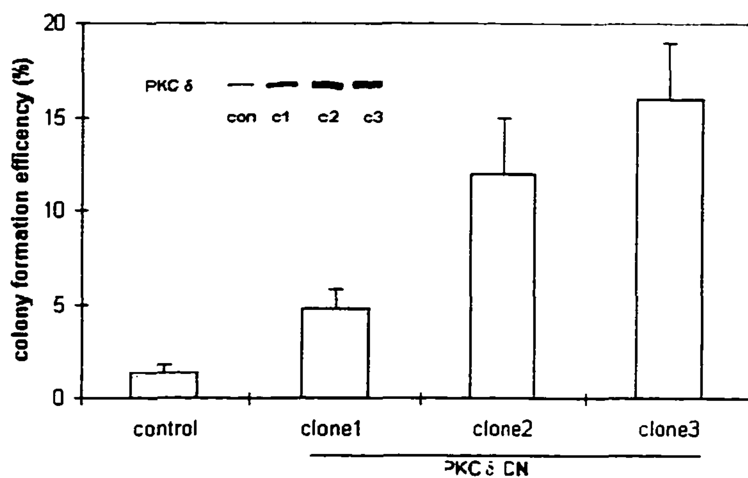

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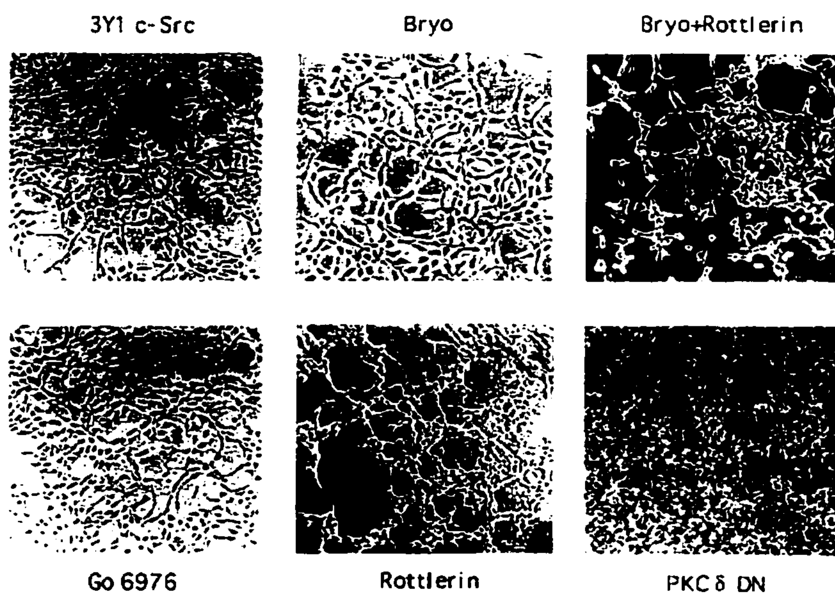

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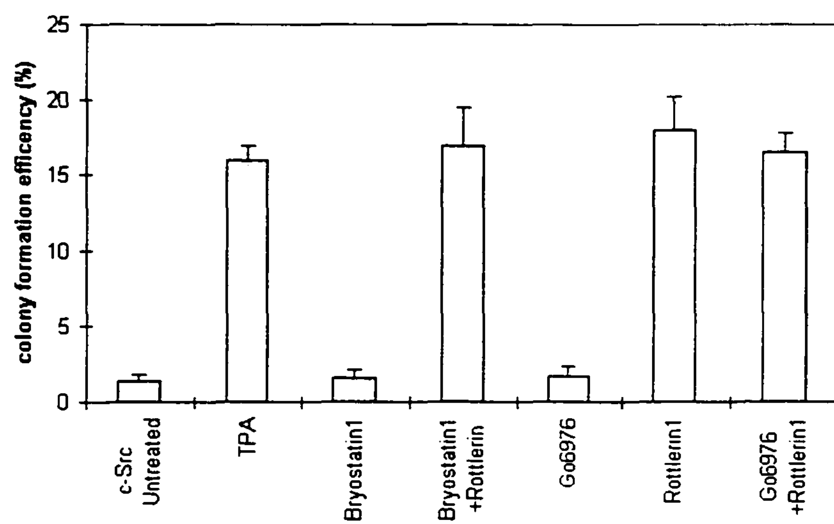

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DISCUSSION

In this chapter, we have shown that the tumor promoting phorbol ester TPA induces a transformed phenotype in 3Y1 rat fibroblasts overexpressing c-Src. The appearance of a transformed morphology in response to TPA treatment correlated with the down-regulation of PKC, especially the α and δ isoforms. Transformation-related phenotypes were also induced by compounds that inhibit PKC. Bryostatin 1 prevented the TPA-induced transformed phenotype in the c-Src-overexpressing cells and the only detectable difference with the PKC isoforms that was observed was that PKC δ was not downregulated by TPA when bryostatin 1 was present. Consistent with PKC δ downregulation being responsible for the effects of TPA, a specific inhibitor of PKC δ and a dominant negative PKC δ mutant both induced anchorage independent growth in cells overexpressing c-Src. These data are consistent with a model in which the tumor promoting effects of phorbol esters is due to the down-regulation of PKC δ.

Several groups have investigated the effects of PKC isoforms on cell growth and PKC isoforms have been reported to have both positive and negative effects upon cell division. The β (Borner et al., (1995) and ε (Mischak et al., 1993) isoforms were reported to stimulate cell growth and transformation. Conversely, the α (Borner et al., 1995) and δ (Mischak et al., 1993; Hirai et al., 1994) isoforms have been reported to be growth inhibitory. In contrast, Liao et al. (1994) reported that overexpression of a truncated PKC δ containing only the regulatory domain inhibited anchorage-independent growth. In the latter report, the authors proposed that their PKC δ regulatory domain could be acting as a

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dominant negative mutant of PKC δ and concluded that PKC δ has a positive role in cell growth. An alternative hypothesis is that overexpression of the PKC δ regulatory domain could have behaved like wild type PKC δ and acted to suppress anchorage-independent growth. Such a mechanism has been demonstrated for the α isoform of PKC where a regulatory domain of PKC α was shown to stimulate PLD activity (Singer et al., 1995). This alternative interpretation would be consistent with the inhibitory role for PKC δ observed here and by others (Mischak et al., 1993; Hirai et al., 1994). It may also be of significance that, unlike the other PKC isoforms, PKC δ can be phosphorylated upon tyrosine (Denning et al., 1993; Li et al., 1994). Tyrosine phosphorylation of PKC δ has been reported be either stimulatory (Li et al., 1994) or inhibitory (Denning et al., 1993). Thus, PKC δ may have different functions in different cellular contexts. The ability to downregulate PKC δ function through tyrosine phosphorylation may be another way to induce the promotion-like effects described here. In this regard, it has been reported that in both v-Ras (1993) and in v-Src-transformed cells (Zang et al., 1997) that PKC δ is tyrosine phosphorylated with a corresponding downregulation of the kinase activity.

It is not clear how downregulation of PKC δ might contribute to transformation. PKC has been reported to down-regulate several receptors with tyrosine kinase activity including the EGF (Decker, 1984; 1985; Downward et al., 1985) and insulin receptors (Chin et al., 1993). And a role for PKC in the down-regulation of receptor tyrosine kinase signals by PKC has been proposed (Seedorf et al., 1995). Thus, it is possible that depleting cells of PKC δ could help sustain an active growth factor-induced signal by preventing down-regulation of an activated receptor. Constitutively active tyrosine

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kinases that lead to transformation have been postulated to function through the transmodulation of receptors with tyrosine kinase activity resulting in ligand-independent activation of these receptors resulting in the generation of cell division signals (Wasilenko et al., 1991; Peterson et al., 1994; Daub et al., 1996). Thus, a possible molecular mechanism for the results presented here is that c-Src activates a receptor tyrosine kinase that is down-regulated by PKC δ. Depleting cells of PKC δ would enhance this signal. This is consistent with the lack of any dramatic effect of TPA treatment upon the kinase activity of c-Src.

The hypothesis that downregulation, rather than activation of PKC is important in tumor promotion is consistent with several studies that have examined the effect of disrupting the interaction between receptor tyrosine kinases and phospholipase Cγ. The PDGF, EGF and FGF receptors all interact with and activate phospholipase Cγ (Noh et al., 1995). Mutations to these receptors that disrupt phospholipase Cγ activation and presumably the subsequent activation of PKC in response to the generated diacylglycerol had little or no effect upon the mitogenic effects of PDGF, EGF, and FGF (Peters et al., 1992; Mohammadi et al., 1992; Decker, 1993; Valius et al., 1993). These studies all suggested that the activation of PKC in response to tyrosine kinase activity is not required for the mitogenic response to these growth factors.

It is not clear to what extent the cell culture model for tumor promotion used here resembles the tumor promoting effects of phorbol esters originally described for mouse skin. However, the effects of TPA on the c-Src-overexpressing cells, in which anchorage-independent growth and amplification of these cells is stimulated, are

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completely consistent with the classical description of a tumor promoter, where initiated cells are amplified by treatment with the tumor promoter. In this regard, it is significant that bryostatin 1, which blocked the promoter-like effects of TPA on the c-Src-overexpressing cells, also prevented the tumor promoting effects of TPA on mouse skin (Hennings et al., 1987). Thus, the promoter-like effects observed here have much in common with tumor promotion in an animal model and suggest that downregulation of PKC δ may also be important for tumor progression.

The ability to stimulate anchorage independent growth with the dominant negative PKC δ mutant and the PKC δ-specific inhibitor suggest that PKC δ has the ability to suppress transformation. In this regard, it is of interest that the PKC δ-specific rottlerin could also stimulate anchorage independent growth of the parental 3Y1 cells, albeit to a substantially reduced level, suggesting that PKC δ may be suppressing transformation of many cells. These data implicate PKC δ as a tumor suppressor and suggest that PKC δ or downstream substrates of PKC δ could be targets for therapeutic intervention.

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CHAPTER IV

Activation of Protein Kinase C Triggers its Ubiquitination and Degradation

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INTRODUCTION

Tumor promotion by phorbol esters involves the selective amplification of cells previously mutated in an appropriate growth stimulatory gene (Dingiovanni, 1992; Yupsa et al., 1988). Phorbol esters exert their effects on the protein kinase C (PKC) family of genes that consists of at least 9 distinct isoforms that are responsive to tumor promoting phorbol esters (Nishizuka, 1995). Phorbol esters first activate, but upon prolonged treatment, phorbol ester-responsive PKC isoforms are proteolytically degraded (Young et al., 1987). Using a cell culture model system in which cells overexpressing c-Src were transformed by phorbol ester treatment, we demonstrated that the tumor promoting effect of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) upon these cells was due to the depletion of PKC δ (Lu et al., 1997). These data suggested that PKC δ may function as a tumor suppressor. Consistent with this hypothesis, PKC δ is inactivated by tyrosine phosphorylation in cells transformed by v-Src (Zang et al., 1997) and v-Ras (Denning et al., 1993). Thus, regulation of PKC δ at the level of activity and expression may be a very important cell growth control mechanism.

PKC α has been reported to become ubiquitinated in response to bryostatin 1, an activator of PKC that prevents tumor promotion in mouse skin by TPA (Lee et al., 1996). The ubiquitin-proteasome pathway is a non-lysosomal degradation system that controls the timed destruction of cell cycle regulatory proteins including the tumor suppressor p53; cyclin-dependent kinase inhibitor p27; the cyclins; the oncogenes c-Myc, c-Jun, c-Fos; and the transcription factors NFKB and E2F (Rolfe et al., 1997).

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The ubiquitin-proteasome pathway involves a three or four step ubiquitin thioester cascade. The highly conserved 76 amino acid ubiquitin molecules is first activated by the ubiquitin-activating enzymes (E1) through formation of a high energy thioester linkage of its C-terminal glycine with cysteine residue in the E1 itself. Subsequently, the ubiquitin moiety is transfered to a cysteine of one of the ubiquitin-conjugating enzymes (UBC or E2), which in turn attaches the ubiquitin part onto a target protein with or without the assistance of a ubiquitin-protein ligase (E3). The latter step may involve a third thioester linkage with the E3 enzyme and results in the formation of an isopepitide bond of the activated C-terminal glycine of ubiquitine with the N-terminus or the e-NH2 group of an internal lysine residue of the substrate polypeptide. Both E2 and E3 proteins belong to large families of proteins and it is believed that different combinations of E2s with different E3 ligases define a high substrate specificity. The monoubiquitinated substrate then undergoes further ubiquitination via the lysine residue at position 48 on the ubiquitin chains (chau. et al, 1989) that target proteins for degradation by the 26S (1500Kda) proteasome complex (Goldberg and Rock,1992, Hershko and Ciechanover, 1992).

The 20S (600 Kda) proteasome particle (multicatalytic protease) comprises the catalytic core of the 26S particle. The proteasome is highly conserved and is present in the cytoplasm and nucleus of all eukaryotic cells. By itself, the 20S proteasome is unable to degrade ubiquitinated proteins but through an ATP-dependent mechanism, it associates with other components to form the 26S proteasome complex capable of hydrolyzing Ubiquitin-conjugated protein (Eytan et al, 1989; Driscoll and Goldberg, 1990).

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In this study, we have investigated the role of the ubiquitin-proteasome pathway in the downregulation of PKC isoforms in response to the tumor promoting phorbol ester TPA.

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RESULTS

Proteasome inhibitors block depletion of PKC isoforms.

To investigate whether the ubiquitin-proteasome pathway is involved in the downregulation of PKC in response to phorbol esters, we first examined the effect of proteasome inhibitors on TPA-induced PKC depletion. MG101 and MG132, which inhibit proteasome function (Palombella et al., 1994; Rock et al., 1994), prevented the TPA-induced depletion of the α, δ, and ε PKC isoforms, the only TPA-responsive isoforms present in these cells (Fig. 11). E64, which shares with MG101 and MG132 the ability to inhibit calpain protease, but not the proteasome, had no effect upon TPA-induced PKC depletion. We also examined the effect of these compounds on PKC ζ, a PKC isoform that is expressed in these cells, but not responsive to phorbol esters (Nishizuka, 1995). And, as shown in Fig. 1, neither MG 101 nor MG 132 had any effect upon PKC ζ. These data implicate the ubiquitin-proteasome pathway in the phorbol ester-induced depletion of PKC.

PKC isoforms become ubiquitinated upon TPA treatment.

The data in Fig. 11 demonstrate that compounds which inhibit proteasome function, inhibit TPA-induced downregulation of PKC. Therefore, it is predicted that the affected PKC isoforms should become ubiquitinated in response to TPA. In Fig. 11, it was also observed that the anti-PKC δ antibody recognized several higher molecular weight

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Fig. 11. **Proteasome inhibitors prevent TPA-induced depletion of PKC.**

3Y1 cells overexpressing c-Src were treated with TPA (400 nM) for the indicated times and PKC depletion was monitored by Western blot analysis as described previously (Lu et al., 1997). The effect of MG101, MG132, or E64 (all 50 μM) was determined by adding these compounds 30 min prior to addition of TPA as shown. The levels of PKC δ, α, ε, and ζ were determined using antibodies specific for these isoforms.

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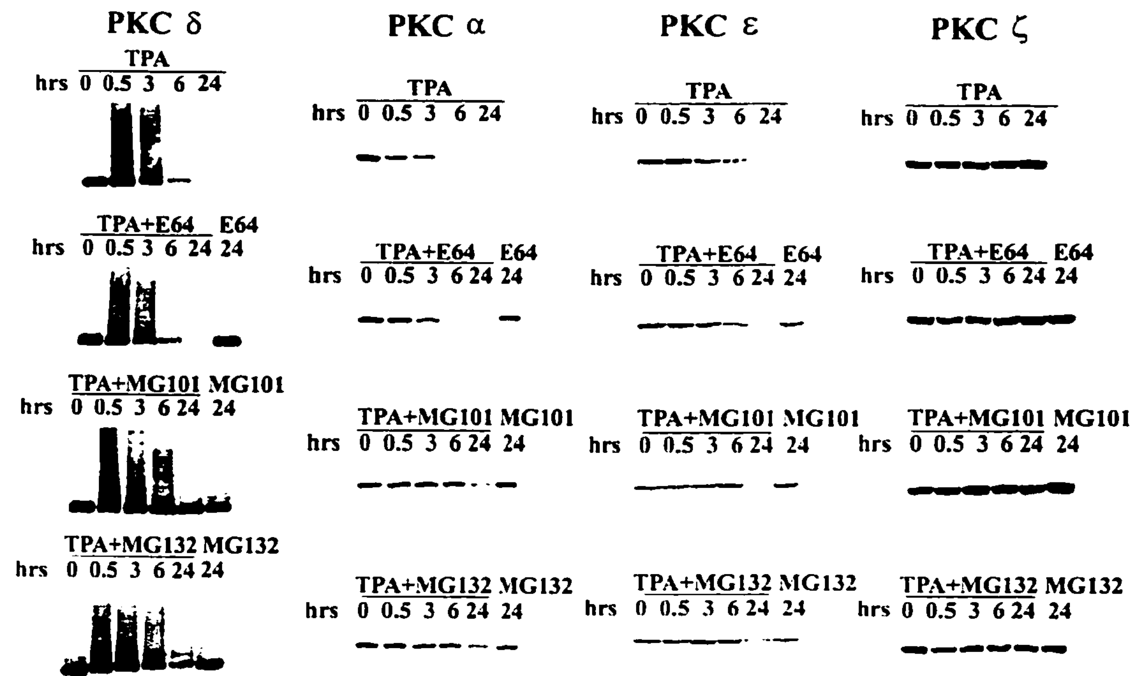

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Fig. 12. **PKC becomes ubiquitinated upon TPA treatment.**

The c-Src-overexpressing 3Y1 cells were treated with TPA (400 nM) for the indicated times. PKC δ, α, ε and ζ were then immunoprecipitated and the level of ubiquitinated PKC was determined by Western blot analysis using an anti-ubiquitin antibody. The effect of MG101 (50 μM) on ubiquitination of untreated cells and cells treated with TPA is shown.

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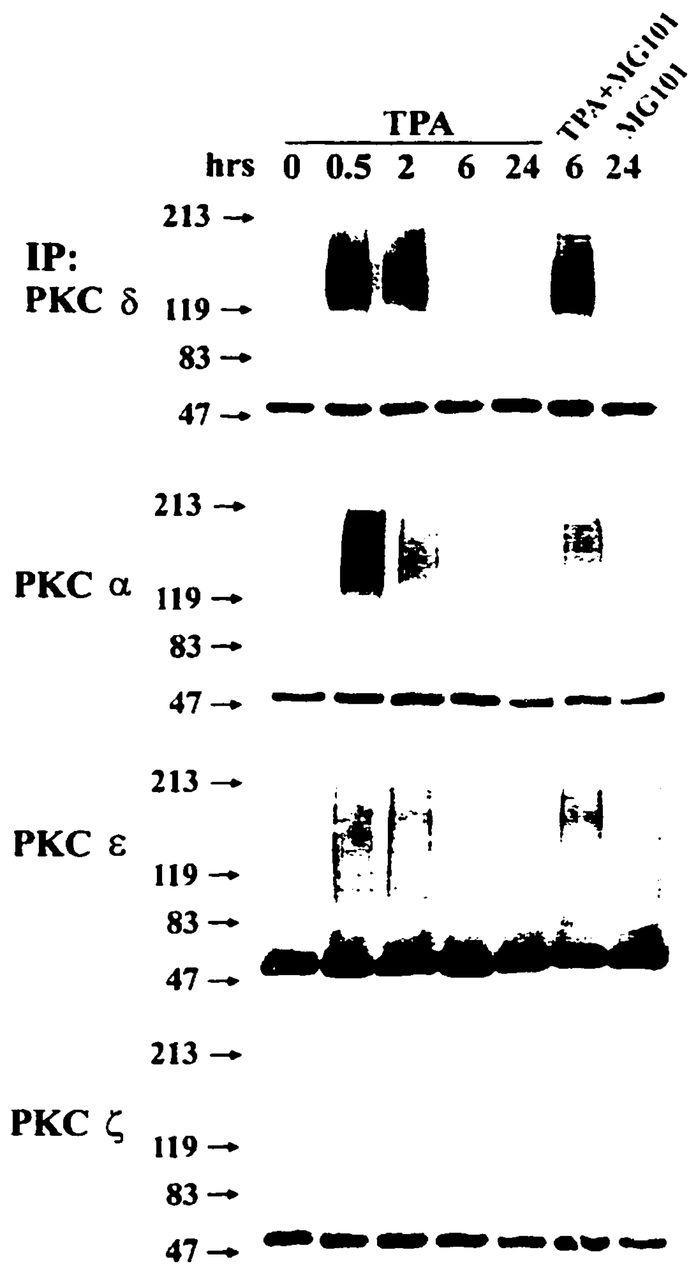

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species within 30 min after TPA treatment. The appearance of these higher molecular weight species of PKC δ is consistent with the rapid ubiquitination of PKC δ in response to TPA. To investigate directly whether PKC isoforms were being ubiquitinated in response to TPA, we performed Western blot analysis of PKC isoform immunoprecipitations using anti-ubiquitin antibody. As shown in Fig. 12, ubiquitination of PKC α, δ, and ε, but not PKC ζ was detected within 30 min of TPA treatment. By 6 hours, the ubiquitinated PKC isoforms were no longer detectable. However, if MG101 was used to inhibit proteasome, then the ubiquitinated isoforms were still present 6 hours after TPA treatment (Fig. 12). Interestingly, 24 hours treatment with MG101 alone resulted in a significant accumulation of ubiquitinated forms of PKC α to a limited extent, and substantially for PKC ε (Fig. 12) suggesting that ubiquitination may occur in response to physiological stimuli as well as TPA. These data demonstrate that PKC isoforms α, δ, and ε rapidly become ubiquitinated in response to TPA treatment and that their disappearance is blocked by inhibiting proteasome.

Degradation and ubiquitination of PKC is dependent upon PKC kinase activity.

To begin to investigate the mechanism for activation of ubiquitination and proteasome degradation, we asked whether the kinase activity of PKC was important for degradation. We first investigated the effect of PKC inhibitors on the TPA-induced PKC downregulation and ubiquitination. In Fig. 13a, it is shown that the PKC inhibitors staurosporine and bisindolylmaleimide II prevented downregulation of PKC isoforms α,

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Fig. 13. **Degradation of PKC is dependent upon PKC kinase activity.**

(A) 3Y1 cells overexpressing c-Src were treated with TPA (400 nM) for 6 hours to deplete the cells of PKC. This was then performed in the presence of the PKC inhibitors staurosporine, bisindolylmaleimide II, Go 6976, and rottlerin at the indicated concentrations and PKC levels were determined by Western blot analysis as in Fig. 11. (B) The effect of the PKC inhibitors on TPA-induced ubiquitination of PKC α and δ was determined as in Fig. 12. (C) The ability of TPA to induce translocation of the PKC isoforms from the cytosol to the membrane in the presence of PKC inhibitors was investigated by Western blot analysis of the PKC isoforms present in the cytosolic and membrane fractions before and after TPA treatment. The effect of the inactive phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD) on the induction of PKC isoform down-regulation (D) and ubiquitination of PKC δ (E) was examined as in A and B respectively.

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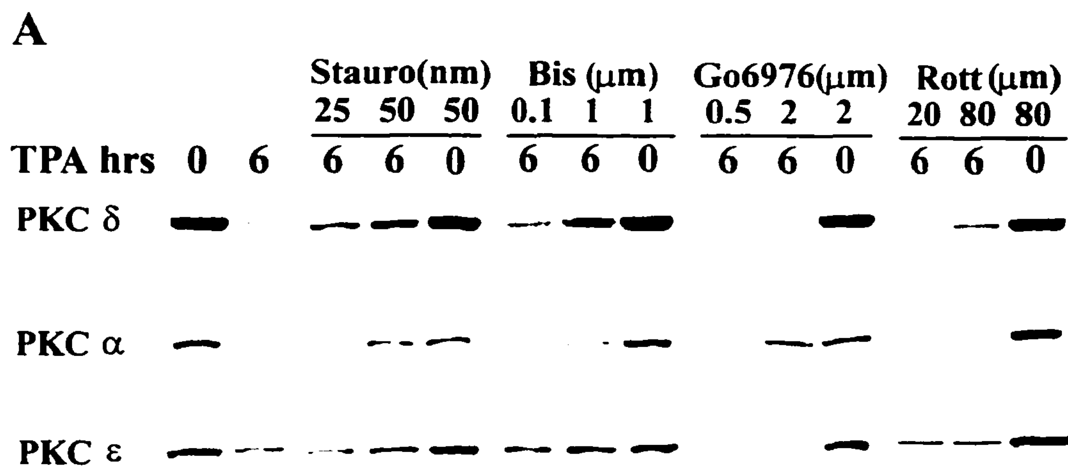

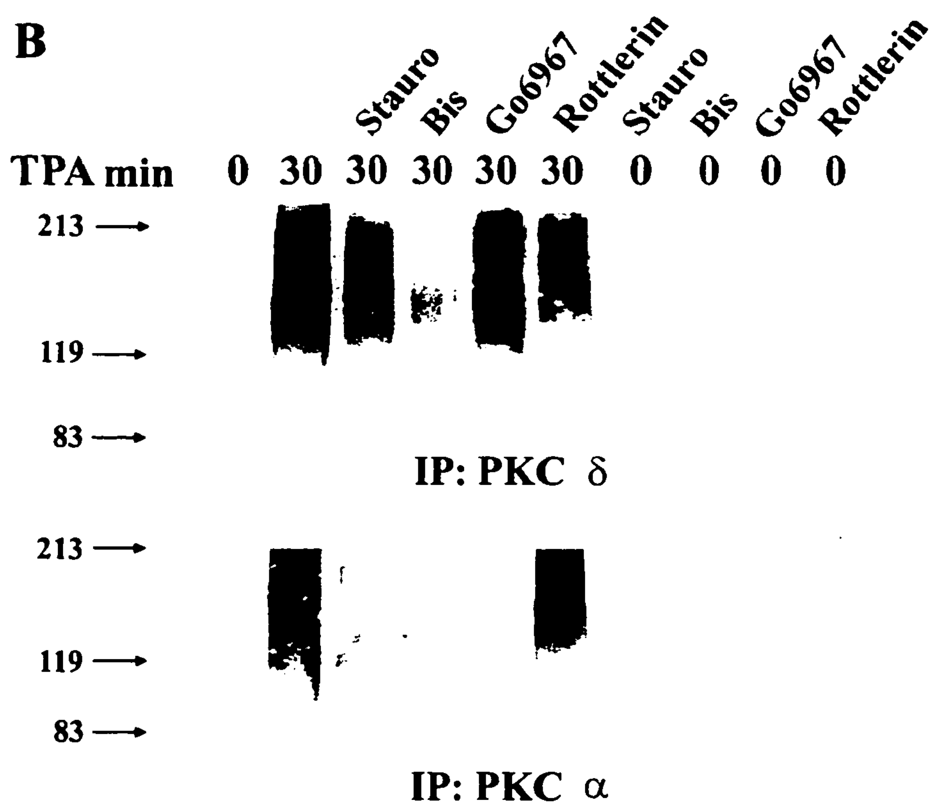

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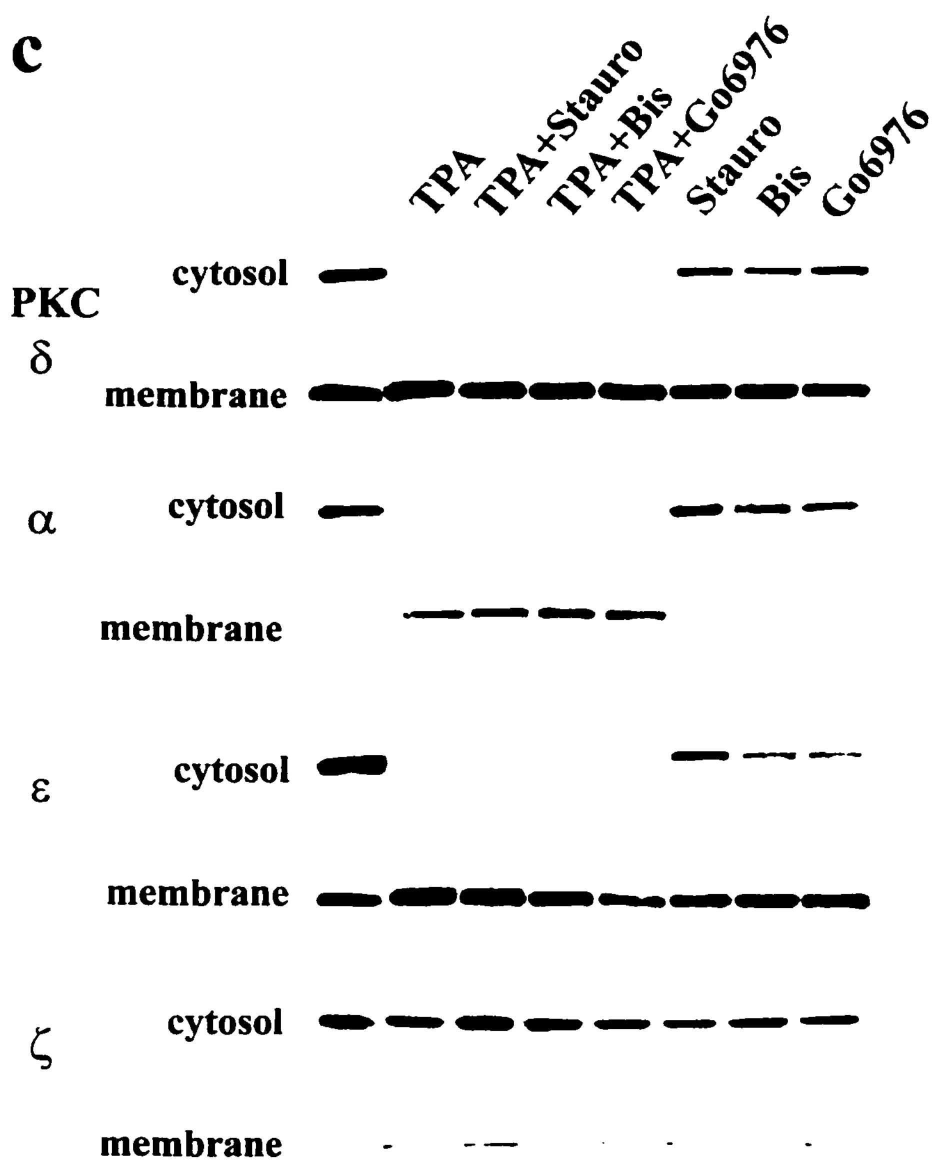

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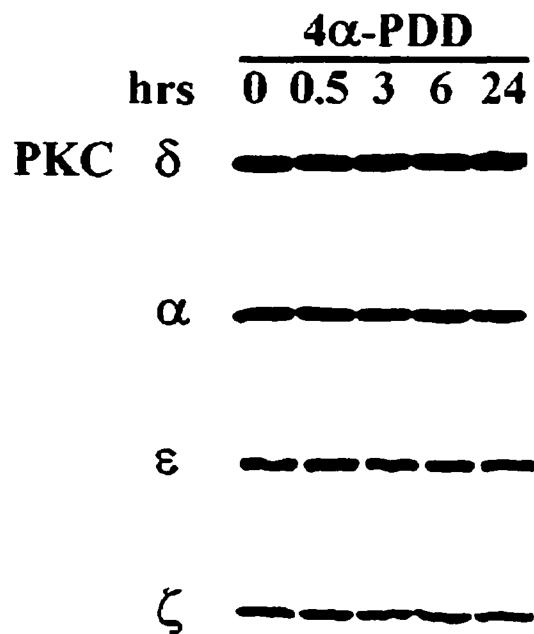

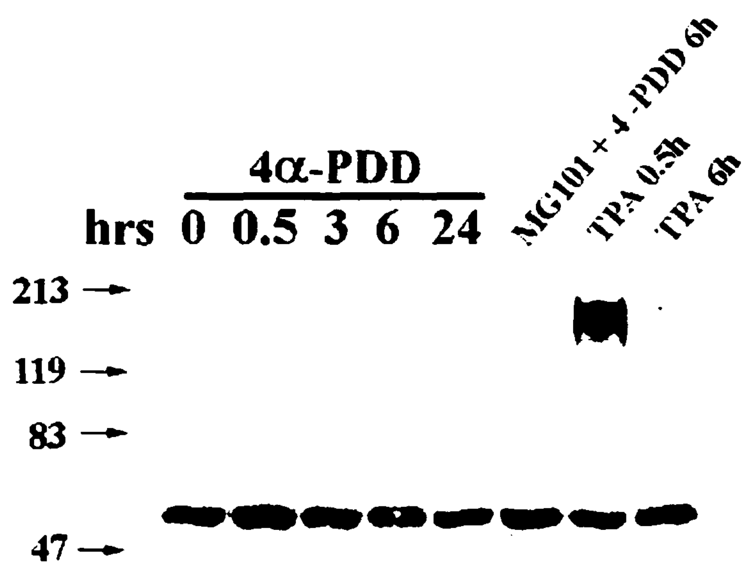

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δ, and ε. Interestingly, Go6976, which specifically inhibits PKC α (Martiny-Baron et al., 1993), prevented TPA-induced downregulation of the α isoform only. And rottlerin, a more specific inhibitor of PKC δ (Gschwendt et al., 1994) prevented TPA-induced downregulation of the δ isoform only. We also investigated the effect of the PKC inhibitors on the ubiquitination of PKC isoforms α and δ, and as expected, the PKC inhibitors also prevented TPA-induced ubiquitination of these PKC isoforms with the same specificity observed for inhibition of downregulation (Fig. 13b). The PKC inhibitors did not inhibit translocation to the membrane of the PKC isoforms (Fig. 13c). Thus, the effects observed in Fig. 13a and 13b were not due to a lack of membrane association. These data indicate that TPA-induced downregulation and ubiquitination of the PKC isoforms requires an active kinase activity. And consistent with a requirement for activation PKC for downregulation, the inactive phorbol ester 4α-phorbol 12,13-didecanoate, which does not activate PKC (Suzuki et al., 1996) did not lead to the down regulation of PKC (Fig. 13D), nor did it result in the ubiquitination of PKC α (Fig. 13E).

If PKC kinase activity is required for downregulation, then a kinase-dead PKC mutant should be resistant to downregulation in response to TPA. An ATP-binding site mutant of PKC α (Ueda et al., 1996) that was kinase-dead was introduced into the c-Src-overexpressing cell line and the ability to downregulate PKC α with TPA was examined. As shown in Fig. 14a, this PKC α mutant was completely resistant to downregulation by TPA. Since the kinase-dead PKC α mutant could still be stimulated to associate with the membrane in response to TPA (Fig. 14b), the lack of degradation was not due to lack of membrane localization. Since the PKC δ and ε were both activated and downregulated in

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Fig. 14. **A kinase-dead mutant of PKC α is not downregulated by TPA.**

The c-Src-overexpressing 3Y1 cells were stably transfected with a mutant PKC α gene which has a mutation in the ATP binding site (15). (a) The mutant PKC-α-overexpressing cells were then treated with TPA (400 nM.) and the levels of PKC α, δ, and ε in these and the parental cells were determined at 6 and 72 hours later as in Fig. 11. (b) The ability of TPA to induce translocation of the PKC isoforms from the cytosol to the membrane in the parental cells and in the cells expressing the kinase-dead PKC α was determined as in Fig. 13c.

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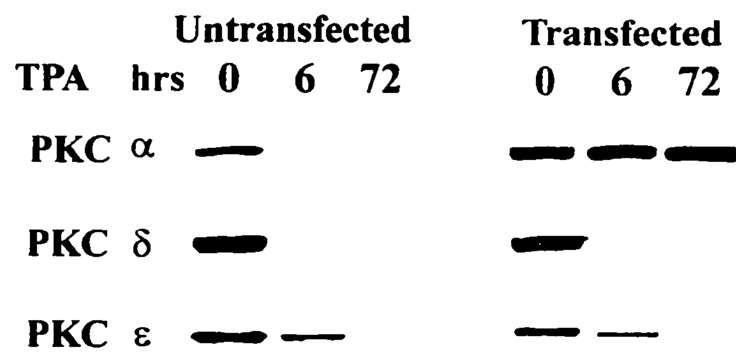

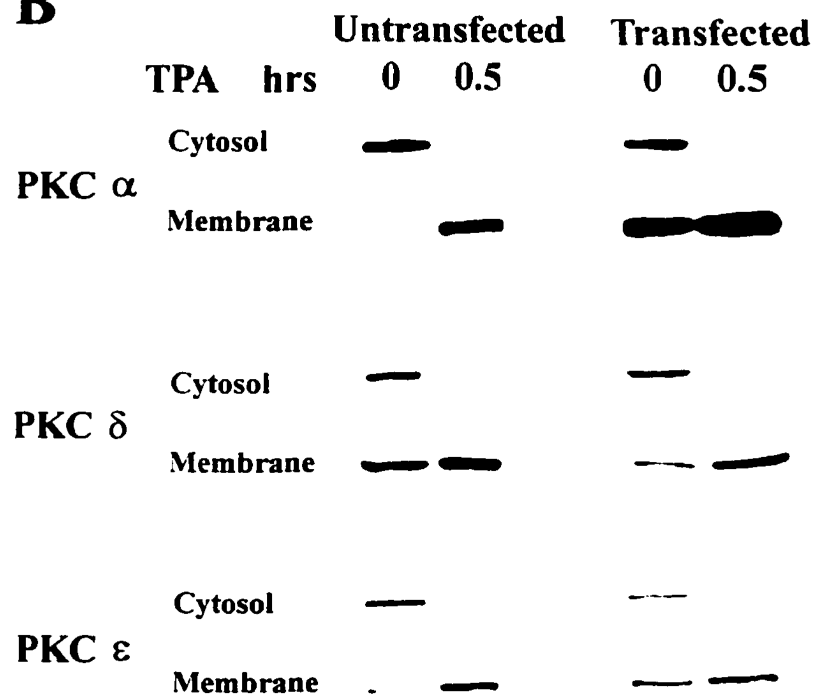

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these cells, activation of the ubiquitin-proteasome pathway by these PKC isoforms was apparently specific for the activated isoforms only. These data further support the conclusion that activating of the kinase activity of PKC is necessary for ubiquitination and downregulation.

PKC is ubiquitinated and downregulated in response to diacylglycerol (DG) in a proteasome and kinase-dependent mechanism.

Phorbol esters bind to PKC at the site that binds the physiological activator DG (Nishizuka, 1996). As shown above in Fig. 12, the proteasome inhibitor MG101 stimulated an increase in ubiquitinated PKC isoforms α and ε, suggesting that ubiquitination is a physiological response and not an artifact of phorbol ester treatment. We therefore wished to investigate whether ubiquitination and downregulation of PKC occurs in response to DG. As shown in Fig. 15, the α and δ isoforms and to a lesser extent the ε isoform were all downregulated in response to the DG dioctoylglycerol (DiC8). This downregulation was sensitive to both proteasome and PKC inhibitors (Fig. 15a). The PKC α-specific Go6976 prevented downregulation of the α isoform specifically. We also wished to determine whether DG stimulated ubiquitination of PKC isoforms. We added DiC8 to the 3Y1 cells and examined ubiquitination as in Fig. 12. In Fig. 15b, it is shown that DiC8 stimulated ubiquitination of PKC δ. The ubiquitination of PKC δ was inhibited by the PKC inhibitors staurosporine, bisindolylmaleimide II and rottlerin, but not by the proteasome inhibitor MG101 or the PKC α inhibitor Go6976 (Fig. 15b). These data suggest that PKC isoforms become ubiquitinated and

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Fig. 15. **PKC is downregulated and ubiquitinated in response to diacylglycerol (DG) in a proteasome and kinase-dependent mechanism.**

(a) c-Src-overexpressing 3Y1 cells were treated with DiC8 (10 μg/ml) for the indicated times in the presence of either E64 (50 μM), MG-101 (50 μM), bisindolylmaleimide II (Bis) (1 μM) or Go 6976 (2 μM) and the levels of PKC α, δ, and ε were then determined by Western blot analysis as in Fig. 11. (b) The effect of DiC8 on the ubiquitination of PKC δ was determined in the presence of the proteasome inhibitor MG101 (50 μM) and the PKC inhibitors staurosporine (Stauro) (50 nM), bisindolylmaleimide II (Bis) (1.0 μM), Go 6976 (2.0 μM), and rottlerin (80 μM) as in Fig. 12.

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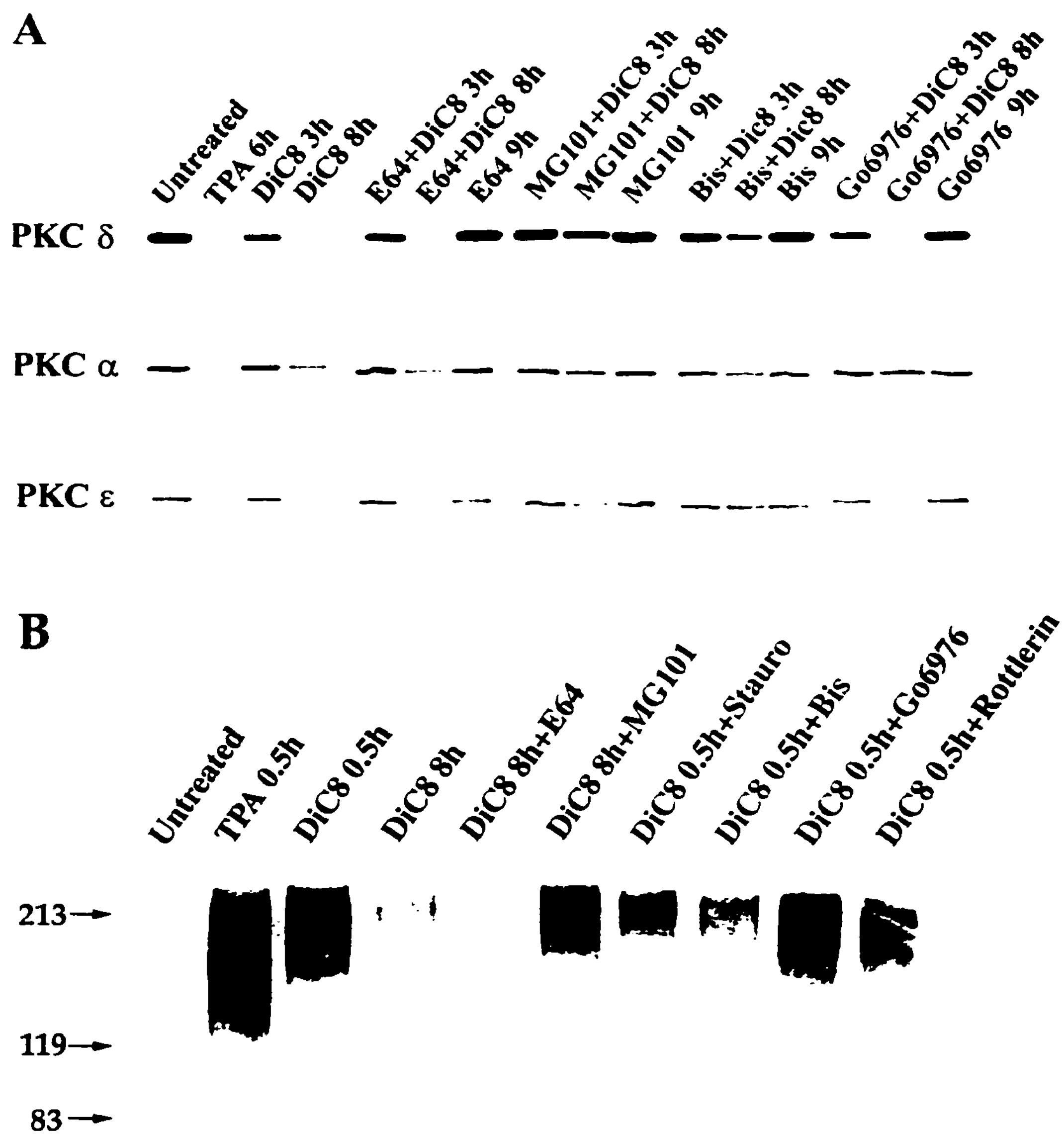

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downregulated by the physiological stimulus of DG as well as by the tumor promoting stimulus of TPA and that downregulation is dependent upon an active kinase.

TPA-induced transformation of 3Y1 cells overexpressing c-Src is blocked by proteasome inhibitors.

In cells overexpressing c-Src, TPA treatment causes the appearance of transformation that is due to the depletion of PKC δ (Lu et al., 1997). We therefore investigated whether inhibitors of the ubiquitin-proteasome pathway could prevent the transformed phenotype induced by TPA in the c-Src-overexpressing cells by preventing the depletion of PKC δ. As shown in Fig. 16a, the proteasome-specific inhibitor MG101 prevented the morphological transformation of the c-Src-expressing cells induced by TPA; whereas, the non-specific protease inhibitor E64 did not prevent the morphological transformation induced by TPA. The proteasome inhibitors had no affect on the transformed phenotype induced by v-Src (Fig. 16a). The ability of MG101 to prevent the TPA-induced morphological transformation was not likely due to any effects that proteasome inhibition has upon cell cycle progression (Pagano et al., 1995), since aphidicolin, which blocks cells at the G1/S boundary of the cell cycle (Johnston, 1986) had no effect upon the TPA-induced morphological transformation (data not shown). In addition, MG101 had no effect upon the translocation of the PKC isoforms induced TPA (Fig. 16b). Thus, the effect observed in 16a is not due to the inability to translocate PKC isoforms to the membrane. These data suggest that PKC δ is downregulated by the ubiquitin-proteasome pathway and that this pathway is critical for the TPA-induced tumor promotion as reported previously (Lu et al., 1997).

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Fig. 16. **TPA-induced transformation of 3Y1 cells overexpressing c-Src is blocked by proteasome inhibitors.**

(a) 3Y1 cells overexpressing c-Src that had been either untreated or treated with TPA (400 nM; 10 hours) in the presence of either MG101 (50 μM) or E64 (50 μM) and the morphology of the cells was examined. The effect of MG101 on v-Src transformed 3Y1 cells is also shown. (b) The ability of TPA to induce translocation of the PKC isoforms from the cytosol to the membrane in the presence of MG101 and E64 was investigated by Western blot analysis of the PKC isoforms present in the cytosolic and membrane fractions before and after TPA treatment.

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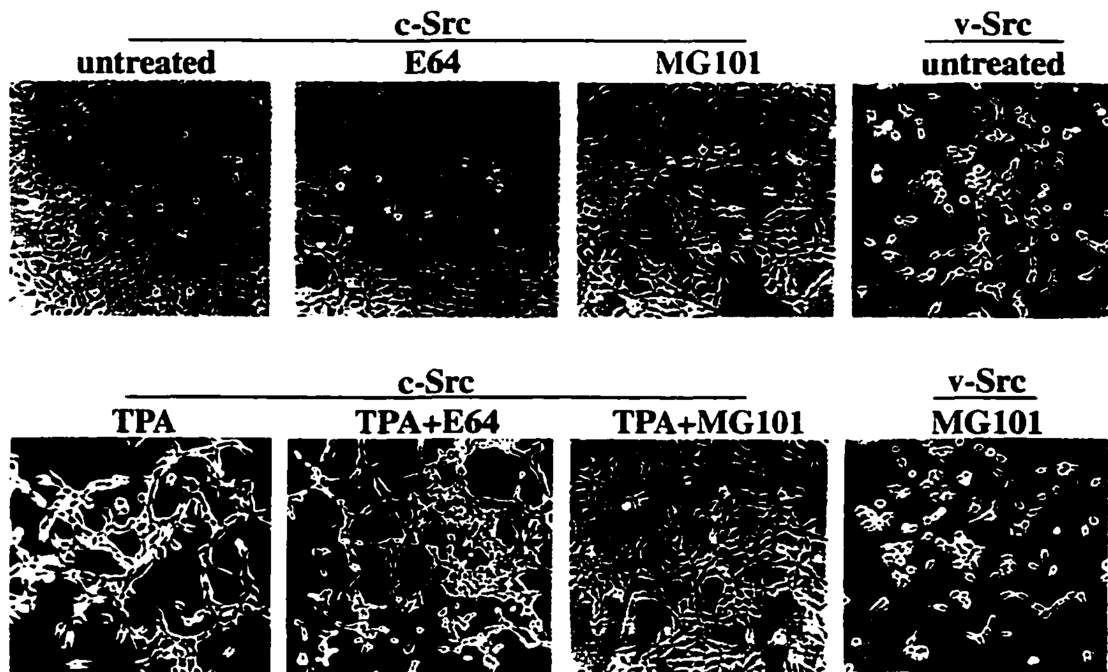

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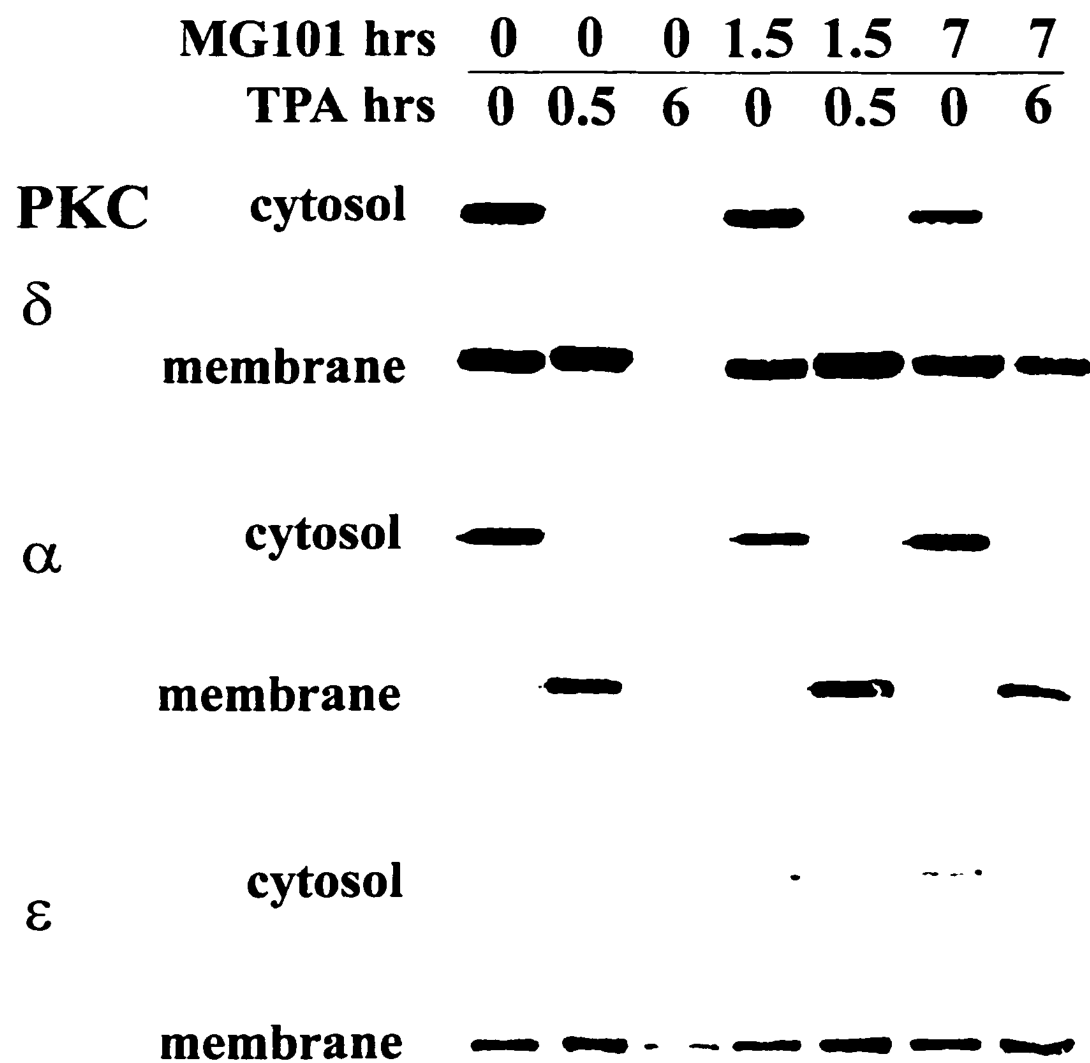

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DISCUSSION

In these experiments, we have shown that downregulation of PKC in response to tumor promoting phorbol esters is via the ubiqutin-proteasome pathway. In response to TPA, PKC isoforms α, δ, and ε all became ubiquitinated with in 30 min and were degraded within 6 hours in 3Y1 rat fibroblasts. Proteasome inhibitors prevented TPA-induced PKC downregulation, but not ubiquitination of the PKC isoforms. Ubiquitination and downregulation of PKC isoforms was dependent upon an active PKC kinase. We previously demonstrated that the downregulation of PKC δ was responsible for the tumor promoting effects of TPA on 3Y1 cells overexpressing c-Src (Lu et al., 1997). Consistent with PKC δ downregulation being important for the tumor-promoting effects observed previously, the proteasome inhibitor MG101, which prevented PKC δ downregulation in response to TPA, also prevented the TPA-induced transformation of the c-Src-overexpressing cells. Thus, the data presented here implicate the ubiquitin-proteasome pathway in phorbol ester-induced tumor promotion.

Interestingly, treatment of 3Y1 cells with MG101 induced the appearance of PKC polyubiquitinated forms, especially for PKC ε, which tends to be the most constitutively activated isoform in these cells (Sang et al., 1995). This suggested that ubiquitination of PKC is a physiological response and not unique to the response to phorbol esters. Consistent with this hypothesis, ubiquitination and downregulation was observed in response to an exogenously provided DG. DG was less potent than TPA at inducing ubiquitination and downregulation of PKC; however, this was most likely because DG

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can be metabolically converted to other lipids such as phosphatidic acid and monoacylglycerol.

The data presented here do not demonstrate the complete mechanism of activation of the ubiquitin-proteasome pathway, however, it is apparently regulated at the level of ubiquitination. Of special interest is the requirement for the kinase activity of the PKC isoforms. Compounds that inhibit activation of PKC prevented PKC downregulation and ubiquitination in response to TPA. Additionally, a kinase-dead PKC α was completely resistant to TPA-induced downregulation. Since phorbol esters still lead to the activation and downregulation of PKC isoforms δ and ε in cells expressing the kinase-dead PKC α, ubiquitination is apparently isoform specific and the activation of one PKC isoform does not stimulate ubiquitination and downregulation of other inactive PKC isoforms. Moreover, since the cells expressing the kinase-dead PKC α likely still express wild type PKC α, which would be activated by TPA, it is not likely that PKC α activates a PKC α-specific ubiquitination system because this would result in the degradation of the kinase-dead PKC α. Since the defect in the kinase-dead PKC α mutant that was not degraded in response to TPA was in the ATP-binding site, activation of the ubiquitin-conjugating system is likely stimulated by a conformational change in PKC that involves ATP binding or hydrolysis. This suggests a suicide model for regulation of PKC where upon activation, PKC becomes ubiquitinated and thereby targeted for degradation in a negative feedback control mechanism.

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CHAPTER V

Summary

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Tumor Promoters, not carcinogenic by themselves, stimulate the formation of tumors when applied along with a carcinogen to experimental animals. Tumors occur only if the exposure to the tumor promoter follows exposure to the initiator which are DNA-damaging carcinogens. The best studied tumor promoter, phorbol ester, exerts its effect on its intracellular receptor, Protein Kinase C. PKC is a multigene family of serine/threonine kinases and nine out of eleven isoforms are responsive to the tumor promoting phorbol ester. Upon phorbol ester treatment, PKC isoforms become associated with membrane and active. However, upon prolonged phorbol ester treatment, PKC is proteolytically degraded. It is not clear whether activation or depletion of PKC is important for the tumor promoting effects of phorbol esters.

We have found that the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulates anchorage independent growth and other transformation-related phenotypes of rat fibroblasts overexpressing the c-Src protooncogene. Thus, TPA is able to induce amplification of cells that have an initiating mutation (c-Src overexpression), and is therefore functioning very much like a tumor promoter in this cell culture model. The appearance of transformed phenotypes induced by TPA in these cells correlated not with activation, but rather with depletion of expressed PKC isoforms. Consistent with this observation, PKC inhibitors also induced transformed phenotypes in the c-Src-overexpressing cells. Bryostatin 1, which inhibited the TPA-induced downregulation of the PKC δ isoform specifically, blocked the tumor-promoting effects of TPA, implicating PKC δ as the target of the tumor promoting phorbol esters. Consistent with this hypothesis, expression of a dominant negative PKC δ mutant in cells

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expressing c-Src caused transformation of these cells; and rottlerin, a protein kinase inhibitor with specificity for PKC δ, like TPA, caused transformation of the c-Src-overexpressing cells. These data implicate that the downregulation of PKC δ results in phorbol ester-induced tumor promotion effect.

To look for the mechanism of phorbol ester-induced downregulation of PKC, we investigated the role of the ubiquitin-proteasome pathway in the downregulation of PKC isoforms in response to the tumor promoting phorbol ester TPA. In 3Y1 rat fibroblasts, proteasome inhibitors prevent the depletion of PKC isoforms α, δ, and ε in response to the TPA. Consistent with the involvement of the ubiquitin-proteasome pathway in the degradation of PKC isoforms, ubiquitinated PKC α, δ, and ε was detected within 30 min of TPA treatment. Diacylglycerol, the physiological activator of PKC, also stimulated ubiquitination and degradation of PKC suggesting that ubiquitination is a physiological response to PKC activation. Compounds that inhibit activation of PKC prevented both TPA- and diacylglycerol-induced PKC depletion and ubiquitination. Moreover, a kinase-dead ATP-binding mutant of PKC α could not be depleted by TPA treatment. These data are consistent with a suicide model whereby activation of PKC triggers its own degradation via the ubiquitin-proteasome pathway. Proteasome inhibitors also blocked the tumor promoting effects of TPA on 3Y1 cells overexpressing c-Src, which results from the depletion of PKC δ, this provides further evidence to support that the tumor promoting effect of phorbol esters is due to depletion of PKC δ, which has an apparent tumor suppressor function.

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Evidence of synergism between various cancer-inducing agents in human carcinogenesis has been discovered, and the initiator-promoter model may be just applicable to humans beings as it is to animal model and tissue culture model system. If so, control of cancer could be achieved through control of either initiators or promoters. The implication that PKC δ may work as a tumor suppressor and its regulation through ubiquitination-proteasome pathway could provide approaches for therapeutic intervention of cancers.

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CHAPTER VI

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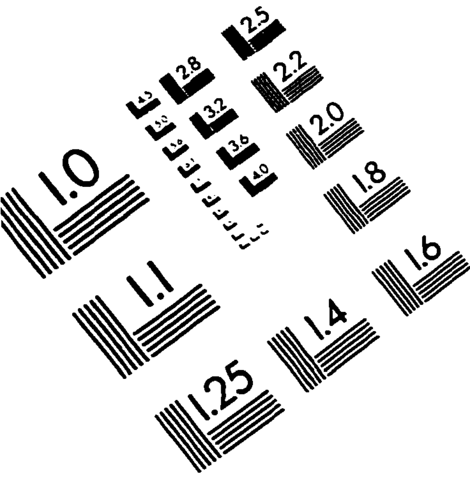
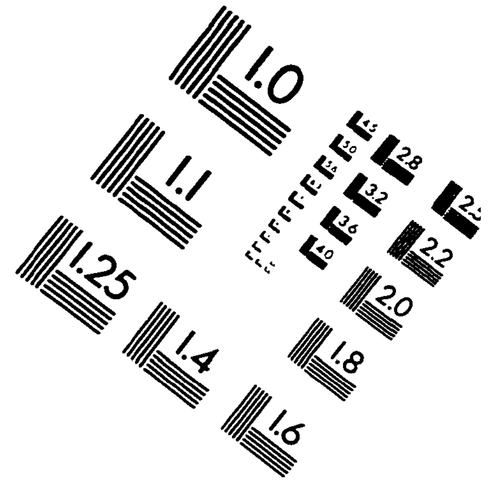
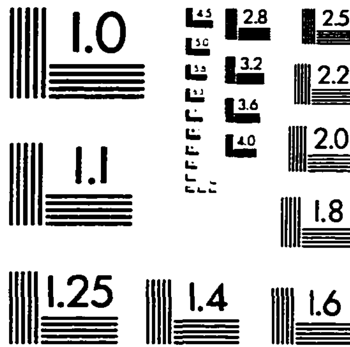
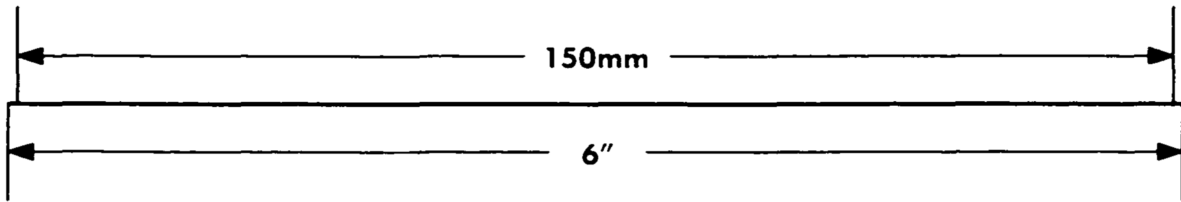
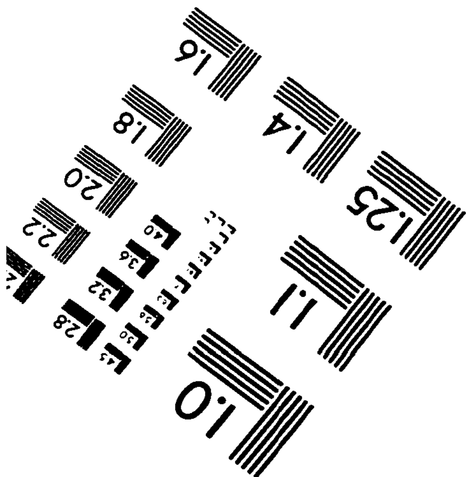
IMAGE EVALUATION TEST TARGET (QA-3)



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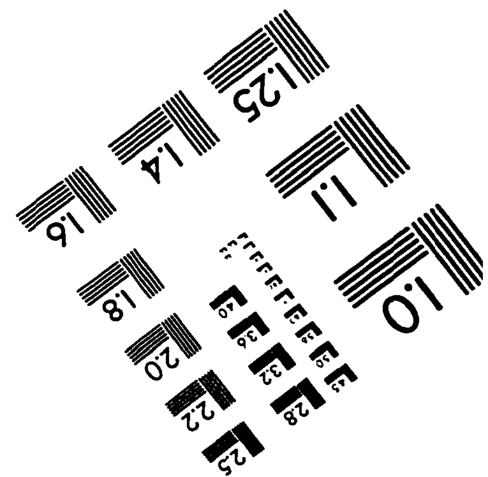

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